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THE INFLUENCE OF HORMONES ON LIPID METABOLISM IN RELATION TO ARTERIOSCLEROSIS

BY

ABRAHAM DURY AND CARLETON R. TREADWELL (*Conference Co-Chairmen*), D. H. BAEDER, N. F. BOAS, R. J. BOUCEK, J. H. BRAGDON, L. COHEN, W. D. COHEN, H. CORBUS, H. FIELD, JR., N. K. FREEMAN, J. W. GOFMAN, T. L. HAYES, N. HIGANO, R. HOPPER, R. J. JONES, J. KALAS, J. E. KIRK, D. LEHR, F. T. LINDGREN, M. I. MULROY, C. W. MUSHETT, A. V. NICHOLS, N. L. NOBLE, Y. T. OESTER, R. W. ROBINSON, R. J. ROSSITER, E. D. SALGADO, J. SEIFTER, E. STAPLE, K. P. STRICKLAND, L. SWELL, E. C. TROUT, JR., M. W. WHITEHOUSE, G. F. WILGRAM, J. F. WOESSNER, JR., AND C. J. D. ZARAFONETIS

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Conference Co-Chairman and Consulting Editor

ABRAHAM DURY

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* This series of papers is the result of a conference on *The Influence of Hormones on Lipid Metabolism in Relation to Arteriosclerosis* held by The New York Academy of Sciences on April 11 and 12, 1958.

INTRODUCTION

Abraham Dury
The Dorn Laboratory for Medical Research of the Glendorn Foundation, Bradford Hospital, Bradford, Pa.

Even a slight familiarity with recent literature on the pathogenesis of atherosclerosis conveys the strong impression that there is a widely held belief that cholesterol and dietary fat are the important factors in the development of this disease. However, what are the basic roles of the cellular and hormonal factors that regulate lipid metabolism and their relation to the pathogenesis of atherosclerosis? With advances in knowledge of the confluent roles of enzymes and hormones in biological processes, these factors can no longer be considered in terms of peripheral or secondary actions bearing on the balance of forces that result in the deposition of lipid in arterial tissue. An even more neglected area of investigation, which may hold the key to arteriosclerosis, is the "ground substance." Since several hormones influence the cellular structure and function of this tissue, there is reason for speculation and study of the role of this tissue in the intrinsic biochemical lesions of the arterial wall. Recent studies of the physicochemical state of lipids in blood have changed some concepts about lipid metabolism; not less significantly, the activity of cellular elements of the ground substance was found to be involved in lipid metabolism and the lipid-transporting mechanism.

The objective of this publication is the provision of information about biological processes germane to the development of arteriosclerosis, since it is time for the presentation, objective evaluation, and integration of our knowledge and ideas of the physiological control and biochemical mechanisms of lipid metabolism. The contributors to this monograph have brought into focus fundamental data that will assist all who are interested in arriving at a better understanding of the processes that may lead to development of atheromatous lesions in the arterial wall.

Part I. Metabolism of Lipids: Biosynthesis, Absorption, Transport

BIOGENESIS OF PHOSPHATIDES AND TRIGLYCERIDES*

R. J. Rossiter and K. P. Strickland†
Department of Biochemistry, University of Western Ontario, London, Canada

Introduction

Atheromatous deposits in the aorta and other blood vessels contain considerable quantities of both phosphatide and triglyceride. Buck and Rossiter[1] summarized the evidence indicating that certain of the phosphatides are major constituents of these intimal deposits in man. The amount of phosphatide was found to increase with increasing severity of the atherosclerotic process.

Weinhouse and Hirsch[2] showed that phosphatides and triglycerides also entered into the atheromatous deposits of rabbits maintained on a diet rich in cholesterol. For this reason, and for the reason that phosphatides may be involved in the absorption, transport and, possibly, the oxidation of triglycerides (see Deuel,[3] Beveridge,[4] and Dawson[5] for reviews), it is appropriate to consider here the general problem of the biological formation of phosphatides and triglycerides.

Isotope experiments indicate that glycerophosphatides may be formed *in vivo* from many different precursors, such as inorganic phosphorus labeled with P^{32}; fatty acids labeled with deuterium, C^{14}, I^{131}, or elaidic acid; acetate labeled with deuterium or C^{14}; choline, ethanolamine, or serine labeled with C^{14} or N^{15}; and glycerol labeled with C^{14}. Similar experiments carried out *in vitro* with tissue slices indicate that for most tissues the glycerophosphatides are formed *in situ* from the appropriate precursors (see Deuel,[6] Dawson,[5] and Rossiter[7] for references)

Chernick *et al.*[8] showed that inorganic P^{32} was incorporated into the phosphatides of slices of rat artery. These experiments demonstrated that blood vessels, like most other tissues, can form phosphatides *in situ* from smaller molecules. More recently, Zilversmit and his colleagues[9, 10] have shown that the major portion of the phosphatides in the aorta of rabbits with atheromatosis caused by a high-cholesterol diet was synthesized *in situ* and was not transported to the aorta from the plasma lipids.

Glycerophosphatides

Chiefly owing to the work of Kennedy,[11, 12] much is known of the metabolic pathways whereby glycerophosphatides, notably lecithin, are formed in liver tissue. Similar metabolic pathways are operative in brain[7, 13] and probably in many other tissues.

Formation of phosphatidic acid. As the result of early *in vivo* experiments[14, 15]

* The work reported in this paper was supported in part by grants from the National Research Council of Canada and the National Mental Health Grants Administration, Ottawa, Canada.

† These studies were performed during the tenure of a Lederle Medical Faculty Award from Lederle Laboratories Division, American Cyanamid Corp., Pearl River, N. Y.

TABLE 1

EFFECT OF COENZYME A ON THE INCORPORATION OF LABELED α-GLYCEROPHOSPHATE INTO THE LIPID OF RAT BRAIN*

Preparation	Precursor	Specific activity (counts/min./μg. P)	
		Control	CoA†
Water homog. (gas, N_2)	α-GP^{32}	13.4	35.5
Mitochondria (gas, O_2)	α-GP^{32}	25.4	89.2
Water homog. (gas, N_2)	α-GP-C^{14}	4.1	10.3

* Conditions of incubation as described previously.[24]
† Final concentration, 5×10^{-5} M.

and later *in vitro* experiments with rat liver mitochondria[16] and cell-free enzyme preparations from rat liver,[17] α-glycerophosphate (α-GP) was implicated as a precursor of tissue glycerophosphatides. The α-GP could arise from the glycolysis intermediate, dihydroxyacetone phosphate, by the action of L-α-GP dehydrogenase,[18-20] or by the transfer of phosphate from adenosine triphosphate (ATP) to glycerol.[21] The enzyme that catalyzes this reaction, glycerokinase, was isolated and partially purified by Bublitz and Kennedy.[22]

When inorganic P^{32} was added to metabolizing cell-free preparations from liver and brain tissue, radioactivity was incorporated into the lipids, but most of this radioactivity was recovered from the phosphatidic acid fraction.[16, 23, 24] Similarly, when α-GP^{32} was used as a precursor, Kennedy[16] and Kornberg and Pricer[17] found that the radioactivity appeared in phosphatidic acid. This incorporation was stimulated by the addition of coenzyme A (CoA), long-chain fatty acids, and ATP.[25]

TABLE 1, taken in part from the results of McMurray *et al.*,[24] shows that the radioactivity of α-GP^{32} and α-GP-C^{14} was incorporated into the lipid of glycolyzing homogenates or phosphorylating mitochondria from rat brain. Examination of the hydrolysis products of the labeled lipid by the method of Dawson[23] showed that for both α-GP^{32} and α-GP-C^{14} most of the radioactivity was in the phosphatidic acid, with negligible activity in the glycerophosphatides. The incorporation was greatly stimulated by the addition of CoA. Moreover, a supply of metabolic energy was required for the labeling of the phosphatidic acid, since the incorporation was abolished either by the addition of inhibitors that prevented the production of ATP (iodoacetate for glycolyzing homogenates and 2:4-dinitrophenol for phosphorylating mitochondria) or by the omission of factors (such as oxidizable substrate and adenine nucleotides) necessary for the continued formation of ATP.

The above experiments are explained by the important contribution of Kornberg and Pricer[26] that guinea pig liver contained an enzyme system capable of activating long-chain fatty acids to form thioesters of CoA, together with the production of adenosine 5′-monophosphate (AMP) and inorganic pyrophosphate (P-P):

$$\text{Fatty acid} + \text{ATP} + \text{CoA} \rightleftharpoons \text{acyl-CoA} + \text{AMP} + \text{P-P} \qquad (1)$$

Presumably, the mechanism of the reaction is similar to that of the acetic thiokinase reaction, which has been the subject of several recent studies.[27, 28]

Kornberg and Pricer[29] also demonstrated the presence of a second liver enzyme system that carried out the esterification of L-α-GP to form L-α-phosphatidic acid:

$$\text{L-}\alpha\text{-GP} + 2 \text{ acyl-CoA} \rightleftharpoons \text{L-}\alpha\text{-phosphatidic acid} + 2 \text{ CoA} \qquad (2)$$

Jedeikin and Weinhouse[30] and Stansly[31] have provided further evidence for such a reaction. The details of the esterification must still be clarified. It is not certain whether the α′- or the β-hydroxyl group of L-α-GP is esterified first.

Glycerol was inactive[29] in an enzyme system similar to that catalyzing REACTION 2 and glycerol-C^{14} was not incorporated into brain lipids in experiments similar to those described in TABLE 1. It thus seems that phosphatidic acid is formed by the esterification of α-GP, but that diglyceride cannot be formed by a similar esterification of glycerol.

Dephosphorylation of phosphatidic acid. The evidence summarized above indicates that in many cell-free preparations the radioactivity of α-GP^{32} is incorporated into phosphatidic acid, but not into the glycerophosphatides. This finding raises the question of the role of phosphatidic acid in glycerophosphatide synthesis. Since it has been shown by Kennedy[12] that D-α,β-diglycerides are intermediates in the formation of both glycerophosphatides and triglycerides (see below), considerable interest has arisen concerning possible sources of D-α,β-diglycerides. Smith *et al.*[32] showed that D-α,β-diglyceride can be formed by the enzymatic dephosphorylation of phosphatidic acid. The enzyme, phosphatidic acid phosphatase, causes the liberation of inorganic P (P_i) from phosphatidic acid:

$$\text{L-}\alpha\text{-phosphatidic acid} \rightleftharpoons \text{D-}\alpha,\beta\text{-diglyceride} + P_i \qquad (3)$$

The enzyme is inhibited by divalent cations such as Mg^{++}. This type of inhibition probably accounts for the accumulation of phosphatidic acid[16, 17, 23, 24, 33, 34] or a "phosphatidic acidlike" compound[35, 36] in many tissue preparations, although only small amounts of phosphatidic acid may be present in the same tissues from freshly killed animals.[37-39]

TABLE 2 shows the hydrolysis of a number of phosphatidic acids by chicken liver and rat brain preparations. It is seen that in general the chicken liver was much more active than the rat brain. With both tissues L-α-dioleyl-GP* was less well hydrolyzed than phosphatidic acids from natural sources. Two samples of natural phosphatidic acid were prepared from lecithin by the action of the phospholipase C of carrot chromoplasts, as outlined by Kates.[40] One sample of lecithin was obtained from ox spinal cord† by the method of Pangborn.[41] The other was a commercial preparation of egg lecithin purified by passage over an alumina column as described by Hanahan *et al.*[42]

* Kindly provided by E. Baer, Banting and Best Department of Medical Research, University of Toronto, Toronto, Canada.

† Extract of spinal cord kindly provided by K. K. Carroll, Department of Medical Research, University of Western Ontario, London, Canada.

TABLE 2
DEPHOSPHORYLATION OF PHOSPHATIDIC ACID*

Tissue	Substrate	Homogenate	Mitochondria plus microsomes
Chicken liver	L-α-Dioleyl-GP		0.5†
	Phosphatidic acid (from egg lecithin)	3.1	2.2
Rat brain	L-α-Dioleyl-GP	0	0.05
	Phosphatidic acid (from spinal cord lecithin)	1.08	—
	Phosphatidic acid (from egg lecithin)	0.87	0.86
	Phosphatidic acid (from egg lecithin) $MgCl_2$ (8 mM)	0.22	0.24

* Incubated at 37° C. in Tris buffer (0.05 M) *p*H 7.4 for 1 hr. Each vessel contained phosphatidic acid, 4 μmoles; 100 mg. homogenate or mitochondria plus microsomes from 300 mg. tissue. Total volume, 3 ml.
† In μmoles, P liberated.

$MgCl_2$, in a concentration (8 mM) similar to that used in the experiments reported in TABLE 1, was inhibitory to the rat brain phosphatidic acid phosphatase.

Formation of lecithin from phosphorylcholine. Early *in vivo* experiments with phosphorylcholine (PCh) gave little support to the supposition that this substance might be an intermediate in the biosynthesis of lecithin.[43] However, interest in PCh was renewed when Kornberg and Pricer[25] showed that PCh labeled with both C^{14} and P^{32} was incorporated, as a unit, into the lipid of a rat liver preparation. Subsequently, Rodbell and Hanahan[44] and McMurray *et al.*[24] showed that P^{32}Ch was incorporated into the lecithin of liver and brain preparations. The earlier claim[45] that choline-C^{14} was incorporated more rapidly than PCh-C^{14} into the lecithin of rat liver mitochondria has not been substantiated.[11]

A great stimulus to the study of the biosynthesis of the glycerophosphatides was the important finding of Kennedy and Weiss[46, 47] that cytidine 5′-triphosphate (CTP) was necessary for the incorporation of P^{32}Ch into lecithin. The requirement was specific for CTP, none of a number of other nucleoside 5′-triphosphates being active. Kennedy and Weiss[47] showed that lecithin was formed according to the following reactions:

$$\text{CTP} + \text{PCh} \leftrightharpoons \text{CMP-PCh} + \text{P-P} \qquad (4)$$

$$\text{CMP-PCh} + \text{D-}\alpha,\beta\text{-diglyceride} \rightleftharpoons \text{L-}\alpha\text{-lecithin} + \text{CMP} \qquad (5)$$

The formation of cytidine diphosphate choline (CMP-PCh) in REACTION 4 represents a novel metabolic role for cytidine nucleotides. PCh is transferred from the intermediate CMP-PCh to a D-α,β-diglyceride acceptor, with the formation of cytidine 5′-monophosphate (CMP). The CMP-PCh was isolated, characterized, and synthesized.[48] The enzyme catalyzing REACTION 4, which was subsequently studied by Borkenhagen and Kennedy,[49] was called PCh-cytidyl transferase and that catalyzing REACTION 5 was called PCh-

TABLE 3

EFFECT OF CYTIDINE TRIPHOSPHATE ON THE INCORPORATION OF LABELED PHOSPHORYLCHOLINE INTO THE LIPID OF RAT BRAIN*

Preparation	Precursor	Specific activity (counts/min./ μg. P)	
		Control	CTP
Water homog. (gas, N_2)	$P^{32}Ch$	3.4	49.8†
Mitochondria (gas, O_2)	$P^{32}Ch$	1.7	31.7‡
Water homog. (gas, N_2)	PCh-C^{14}	1.1	9.3†

* Conditions of incubation as described previously.[24]
† Final concentration of CTP, 7×10^{-4} M.
‡ Final concentration of CTP, 3.5×10^{-4} M.

TABLE 4

EFFECT OF D-α,β-DIGLYCERIDE ON THE INCORPORATION OF LABELED CYTIDINE DIPHOSPHATE CHOLINE INTO THE LIPID OF RAT BRAIN*

Preparation	Precursor	Specific activity (counts/min./ μg. P)	
		Control	D-α,β-Diglyceride
Water homog. (gas, N_2)	CMP-$P^{32}Ch$	35.0	54.8†
Mitochondria (gas, O_2)	CMP-$P^{32}Ch$	48.6	81.0†
Water homog. (gas, N_2)	CMP-PCh-C^{14}	28.0	44.1‡
Water homog. (gas, N_2)	CMP-PCh-C^{14}	25.0	36.8†

* Conditions of incubation as described previously.[50]
† D-α,β-Diglyceride from egg lecithin (1 μmole).
‡ D-α,β-Diglyceride from spinal cord lecithin (1 μmole).

glyceride transferase. Similar enzymes are also present in brain[13, 50] and seminal vesicle.[51]

TABLE 3 shows that the radioactivity of P^{32}- and C^{14}-labeled PCh was incorporated into the phosphatide of glycolyzing homogenates and phosphorylating mitochondria from rat brain. Choline-C^{14} was not incorporated. For both $P^{32}Ch$ and PCh-C^{14}, examination of the labeled lipid by the method of Dawson[23] indicated that the greater part of the radioactivity was in the lecithin. It would seem that the labeling occurred by way of REACTIONS 4 and 5, since activity was dependent upon the presence of CTP. Again, the provision of metabolic energy was found to be necessary for incorporation.

Further evidence that in brain preparations lecithin is formed by REACTION 5 is presented in TABLE 4. In these experiments the radioactivity of P^{32}- and C^{14}-labeled CMP-PCh, synthesized by the method of Kennedy,[48] was incorporated into the phosphatide of rat brain preparations. The incorporation was stimulated by the addition of emulsified D-α,β-diglyceride preparations. The diglycerides were prepared enzymatically, either from egg lecithin or from spinal cord lecithin, by the method of Hanahan and Vercamer,[52] using the lecithinase D of *Clostridium perfringens*.* Examination of the labeled

* Supplied by Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

lipid again showed that lecithin was the only glycerophosphatide containing any significant radioactivity.

Of a number of synthetic α,β-diglycerides* tested, D-diolein was the only one that approached the activity of the natural diglycerides. D-Dimyristin, D-dipalmitin and L-diolein were without effect. This lack of activity of the saturated synthetic D-α,β-diglycerides may be due to their greater insolubility, or to the fact that saturated diglycerides are poor acceptors of PCh for reasons of chemical specificity rather than for physical reasons. The inactivity of L-diolein is not unexpected since the D-configuration is necessary in order for the diglyceride to give rise to glycerophosphatides having the natural L-α-configuration.[53]

There is good evidence that PCh, the precursor of CMP-PCh, can arise in most tissues. Wittenberg and Kornberg[54] described and partially purified an enzyme, which they called choline phosphokinase, that catalyzed the transfer of phosphate from ATP to choline. The enzyme, prepared from yeast, was said to be present in a number of mammalian tissues. Berry *et al.*[55] showed that a similar enzyme was present in extracts of acetone-dried powders from brain and peripheral nerve.

Evidence for the participation of phosphatidic acid in the biosynthesis of lecithin. The low concentration of phosphatidic acid in fresh tissues[38, 39] and in liver slices[56] has led to the suggestion that phosphatidic acid may not be an intermediate in the formation of glycerophosphatides. However, Rodbell and Hanahan[44] reported that the addition of phosphatidic acid increased the incorporation of P^{32}Ch into the lecithin of guinea pig liver mitochondria. Subsequently, Smith *et al.*[32] showed that the addition of phosphatidic acid to a rat liver preparation caused an increase in the incorporation of CMP-PCh-C^{14} into lecithin, provided the phosphatidic acid was first incubated in an Mg^{++}-free medium to allow dephosphorylation to diglyceride.

Experiments in our laboratory with homogenates of rat brain and rat liver mitochondria have provided further evidence for the view that phosphatidic acid is an intermediate in the biosynthesis of glycerophosphatides. TABLE 5 shows the effect of added phosphatidic acid on the incorporation of the radioactivity of CMP-PCh-C^{14} into the lipids of a rat brain homogenate. Before the addition of the CMP-PCh-C^{14}, the phosphatidic acid was preincubated with the homogenate in an Mg^{++}-free medium to allow dephosphorylation to diglyceride according to REACTION 3. It is noted that the presence of Mg^{++} (8 mM) in the preincubation medium greatly decreased the stimulation of the incorporation brought about by the addition of the phosphatidic acid.

In experiments with cell-free preparations from both brain (TABLE 1) and liver,[16, 17] phosphatidic acid is labeled from α-GP^{32}, but none of the radioactivity is incorporated into the glycerophosphatides because, as shown above, the radioactivity is lost as inorganic P^{32} when the phosphatidic acid is converted into diglyceride. TABLE 1 shows that C^{14}-labeled α-GP also was incorporated into the lipid of a rat brain homogenate. Examination of the labeled lipid

* Supplied by E. Baer, Banting and Best Department of Medical Research, University of Toronto.

TABLE 5

EFFECT OF PHOSPHATIDIC ACID ON THE INCORPORATION OF CYTIDINE DIPHOSPHATE CHOLINE-C^{14} INTO LIPIDS OF RAT BRAIN HOMOGENATE*

Conditions†	Specific activity‡	Increase (percentages)
No additions	15.1	—
Phosphatidic acid (from egg lecithin)	32.6	116
Phosphatidic acid (from egg lecithin) + $MgCl_2$ (8 mM)	26.0	48
Phosphatidic acid (from spinal cord lecithin)	54.5	260

* Water homogenate of rat brain (100 mg.) preincubated in 0.05 M Tris buffer *p*H 7.4 for 60 min. at 37° C. in the presence or absence of phosphatidic acid (4 μmoles). At end of 1 hr. $MgCl_2$ (final concentration, 8 mM), Tween 20 (0.25 mg./ml.), and CMP-PCh-C^{14} (0.5 μmole) were added and incubation continued for another 60 min.

† Refers to the conditions of preincubation.

‡ Counts/min./μg. P of sample to which no phosphatidic acid has been added.

TABLE 6

EFFECT OF CYTIDINE DIPHOSPHATE CHOLINE ON THE INCORPORATION OF α-GLYCEROPHOSPHATE-C^{14} INTO THE PHOSPHOLIPIDS OF RAT LIVER MITOCHONDRIA*

Expt. No.		Specific activity of phospholipid (counts/min./μg. P)	Radioactivity (counts/min.)	
			Phosphatidic acid	Lecithin
1	Control	51.4	187	91
	CMP-PCh†	84.2	124	319
2	Control	42.7	44	166
	CMP-PCh†	74.5	33	489

* Conditions of incubation as described previously.[24]

† At 0.5 μmole CMP-PCh per vessel.

again revealed that most of the radioactivity was in phosphatidic acid, and that negligible amounts were in the lecithin. Presumably in this brain system there was sufficient $MgCl_2$ in the medium to prevent the dephosphorylation of the newly formed phosphatidic acid, now labeled in the glycerol portion of the molecule. However, this was not so with preparations from rat and chicken liver. TABLE 6 shows that in rat liver mitochondria α-GP-C^{14} was incorporated into both the phosphatidic acid and the lecithin. Even when Mg^{++} was present in the medium there was sufficient dephosphorylation of phosphatidic acid to enable lecithin labeling to proceed.

TABLE 6 also shows that when unlabeled CMP-PCh was added to rat liver mitochondria capable of incorporating the radioactivity of α-GP-C^{14} into lecithin, the specific activity of the total phospholipid was increased. It is of interest to note that the addition of the CMP-PCh caused a slight decrease in the total number of counts recovered in the phosphatidic acid fraction and a considerable increase in the counts recovered in the lecithin. In this experiment the phosphatidic acid is, no doubt, labeled by way of REACTION 2, and the lecithin is labeled by way of REACTIONS 3 and 5. The addition of an

TABLE 7

INCORPORATION OF RADIOACTIVITY OF LABELED CYTIDINE DIPHOSPHATE CHOLINE AND CYTIDINE DIPHOSPHATE ETHANOLAMINE INTO THE LIPID OF RAT BRAIN HOMOGENATE*

	Specific activity (counts/min./μg. P)	
	CMP-P^{32}-Ch	CMP-P-ethanolamine-C^{14}
Lecithin†	77.3	0
Phosphatidyl ethanolamine	2.7	29
Phosphatidyl serine	1.0	0
Sphingomyelin‡	6.5	—
Phosphatidic acid	0	0
Inositol lipid	0	0

* Conditions of incubation as described previously.[50]
† Hydrolysis products of labeled lipid separated by the method of Dawson.[23]
‡ Lipid stable to the alkaline hydrolysis procedure of Schmidt *et al.*[57]

excess of CMP-PCh has aided REACTION 5 from left to right, with a consequent increase in the labeling of lecithin.

Other glycerophosphatides. It seems probable that the reaction sequence described above for the biosynthesis of lecithin is general, and that similar reactions are involved in the formation of at least some of the other glycerophosphatides. For instance, Kennedy and Weiss[47] showed that an enzyme preparation from chicken liver catalyzed the incorporation of the radioactivity of CMP-P-ethanolamine into phosphatidyl ethanolamine, indicating that this phosphatide is formed by a process involving the cytidine nucleotides.

The data reported in TABLE 7 strengthen this conclusion. The radioactivity of CMP-P-ethanolamine-C^{14} was found to be incorporated into the phosphatidyl ethanolamine of rat brain homogenates. No other fraction examined contained radioactivity.

Suitable precursors occur in most tissues for the formation of other glycerophosphatides. Ethanolamine can be phosphorylated in brain[58] and yeast.[54] Also, Ichihara and Greenberg[59] have described a pathway whereby phosphoserine may be formed from 3-phosphoglyceric acid, a glycolysis intermediate.

Inositol Lipids

In recent years considerable interest has been shown in the inositol lipids. TABLE 8, taken in part from the data of McMurray *et al.*,[24] shows that the incorporation of inorganic P^{32} into the inositol lipid of glycolyzing homogenates and phosphorylating mitochondria from rat brain was stimulated by the addition of CTP. In addition, TABLE 8 shows that the incorporation of inositol-C^{14}, obtained from rye seedlings grown in an atmosphere of $C^{14}O_2$, also was dependent upon the presence of CTP. Agranoff *et al.*[60] have shown that cytidine nucleotides are necessary for the incorporation of *myo*inositol-2-H^3 into the inositol lipids of cell-free preparations of guinea pig kidney.

These findings do not necessarily indicate that inositol lipids are formed by a reaction sequence similar to that outlined above for lecithin. In fact, they may indicate the contrary. However, the results do underline the importance

TABLE 8

EFFECT OF CTP ON THE INCORPORATION OF LABELED PRECURSORS INTO THE INOSITOL LIPID* OF RAT BRAIN†

Preparation	Precursor	Specific activity (counts/min./μg. P)	
		Control	CTP‡
Water homog. (gas, N_2)	Inorg. P^{32}	1440	2430
Mitochondria (gas, O_2)	Inorg. P^{32}	530	1860
Water homog. (gas, N_2)	Inositol-C^{14}	0.4	8.7
		0.7	9.2

* Hydrolysis products of labeled lipid separated by the method of Dawson.[23]
† Conditions of incubation as described previously.[24]
‡ Final concentration, 7×10^{-4} M.

of cytidine nucleotides in the biogenesis of inositol lipids of both brain and other tissues.

Sphingomyelin

Weinhouse and Hirsch[61] reported that the phospholipid obtained from the atheromatous deposits of human aortas was largely ether-insoluble. Buck and Rossiter[1] showed that a considerable quantity of this phospholipid was stable to the alkaline hydrolysis procedure of Schmidt *et al.*[57] It is thus possible that this lipid is sphingomyelin or a similar diaminophosphatide.

Sribney and Kennedy[62] described the presence in chicken liver of an enzyme, PCh-ceramide transferase, that catalyzed the formation of sphingomyelin by the transfer of PCh from CMP-PCh to *N*-acylsphingosine (ceramide):

$$\text{CMP-PCh} + \textit{N}\text{-acylsphingosine} \rightleftharpoons \text{sphingomyelin} + \text{CMP} \qquad (6)$$

REACTION 6 is analogous to REACTION 5 catalyzed by PCh-glyceride transferase. *N*-acylsphingosine rather than D-α,β-diglyceride is the acceptor for the PCh. The formation of CMP-PCh by REACTION 4 has been described above. In addition, Brady and Koval[63] and Zabin[64] have reported that cell-free preparations from rat brain can form sphingosine and ceramide, respectively.

According to Sribney and Kennedy[65] the substance that accepts PCh in REACTION 6 is an "active ceramide." The PCh-ceramide transferase is highly specific, both for CMP-PCh and "active ceramide." Ceramides with short-chain fatty acids in amide linkage are much more active than those with long-chain fatty acids, presumably because the short-chain compounds are more soluble. Sribney and Kennedy[65] summarized the evidence for the conclusion that the sphingosine moiety of the "active ceramide" is threo-1,3-dihydroxy-2-amino-4-*trans*-octadecene, rather than the corresponding erythro compound, which is a constituent of some naturally occurring sphingolipids.[66]

TABLE 7 shows that in a rat brain homogenate a fraction designated as sphingomyelin received some of the radioactivity from CMP-P^{32}Ch. This fraction was stable to the alkaline hydrolysis procedure of Schmidt *et al.*[57]

and so contained the sphingomyelin, which presumably was labeled by a mechanism similar to that of REACTION 6. The presence in this fraction of a phosphosphingolipid that does not contain choline, as has been suggested by work from a number of laboratories,[67-70] would require the revision upward of the specific activity of sphingomyelin.

Triglyceride

Because of the current interest in the effect of dietary triglycerides on cholesterol metabolism, particularly in relation to the development of atheroma, some mention will be made of the biosynthesis of triglycerides. The data of McArthur,[71] incidentally, indicate that the intima of atheromatous aortas contains considerable quantities of triglyceride.

Surprisingly, little is known of the biosynthesis of triglycerides. Borgström[72] showed that it was possible to form triglyceride from fatty acid and diglyceride by reversal of the action of pancreatic lipase. A further suggestion that this method of triglyceride formation may be of importance was made by Jedeikin and Weinhouse,[30] who reported that palmitate-1-C^{14} was incorporated into the triglyceride of slices and cell-free preparations of a number of tissues, without the requirement of a coupled energy source.

That the formation of triglyceride may occur by a pathway different from a reversal of hydrolysis was suggested by the work of Tietz and Shapiro.[73] These workers showed that the incorporation of palmitate-1-C^{14} into tryglyceride of a rat liver homogenate was dependent upon the presence of ATP in the medium. Subsequently Stein and Shapiro[74] reported that this incorporation was greatly accelerated by the addition of α-GP (not free glycerol) and CoA. In addition, they demonstrated that the radioactivity of α-GP-C^{14} was incorporated into triglyceride.

Recently Weiss and Kennedy[75] showed that mitochondria from chicken liver contained an enzyme system capable of forming triglyceride from D-α,β-diglyceride and palmitoyl-CoA, according to the following general equation:

$$\text{Acyl-CoA} + \text{D-}\alpha,\beta\text{-diglyceride} \rightarrow \text{triglyceride} + \text{CoA} \qquad (7)$$

For the formation of triglyceride the acyl-CoA is formed according to REACTION 1, as described by Kornberg and Pricer,[26] and the D-α,β-diglyceride is formed according to REACTIONS 2[29] and 3.[32]

In the experiments reported in TABLE 6 it was shown that the radioactivity of α-GP-C^{14} was incorporated into both the phosphatidic acid and the lecithin of rat liver mitochondria. TABLE 9 shows that in such experiments there was even more radioactivity in the acetone-soluble lipid. That this radioactivity was not due to the presence of phosphatide remaining after the acetone precipitation is shown in the second experiment reported in TABLE 9 in which CMP-PCh-C^{14} was the source of the radioactivity. In this instance, although there was good labeling of the phosphatide, only negligible radioactivity was observed in the acetone-soluble lipid, indicating no gross contamination of this fraction with lecithin.

It thus appears that D-α,β-diglycerides are intermediates in the formation

TABLE 9

INCORPORATION OF C^{14}-LABELED α-GLYCEROPHOSPHATE AND CYTIDINE DIPHOSPHATE CHOLINE INTO THE LIPID OF RAT LIVER MITOCHONDRIA*

Precursor	Acetone-insoluble lipid (phospholipid)	Acetone-soluble lipid (chiefly triglyceride)
α-GP-C^{14}	689† 753	2210† 1340
CMP-PCh-C^{14}	2320 1690	24 40

* Conditions of incubation as described previously.[24, 50]

† Counts per min. per sample. Subsequent examination of the fraction by silicic acid chromatography revealed that much of the activity is present in a phosphorus-containing lipid that is readily eluted by methanol. Mild alkaline hydrolysis and chromatographic separation of this material yielded a spot corresponding to GP.[23] A small but significant portion of the activity remained in the triglyceride fraction. This activity was reduced by the presence of CMP-PCh in the reaction mixture.

of both lecithin and triglyceride. This does not mean that both lecithin and triglyceride are derived from a common metabolic pool of diglyceride. The diglycerides destined to become triglycerides may differ considerably from those destined to become glycerophosphatides, particularly in the nature of their constituent fatty acids. Hanahan[76] has pointed out that each of a series of lecithins from different sources has its characteristic arrangement of fatty acids. Also, there is good evidence that each of the fatty acids of the lecithin molecule is formed by a characteristic metabolic process.[77]

It has been reported that the *in vitro* incorporation of certain precursors into the fatty acids of phospholipid is quite sensitive to metabolic inhibitors[30] and reduced oxygen tensions,[30, 78] whereas the incorporation of the same precursors into triglyceride is less sensitive. This is, perhaps, not surprising. In the formation of both glycerophosphatide and triglyceride, fatty acid must be activated as in REACTION 1 but, in the formation of glycerophosphatide, choline also must be activated as in REACTION 4. For this, an additional source of ATP is necessary for the maintenance of adequate concentrations of CTP. Presumably the CTP is regenerated by the rephosphorylation of the CMP formed in REACTION 5 by enzymes similar to those described by Herbert and Potter.[79]

Summary

Tissue slice experiments have demonstrated that in most tissues, including blood vessels, phosphatides can be formed *in situ* from suitable precursors.

Evidence is given for the conclusion that in cell-free enzyme systems D-α,β-diglyceride, derived from L-α-phosphatidic acid, is an intermediate in the formation of lecithin. Cytidine triphosphate is necessary for the enzymatic transfer of phosphorylcholine to the D-α,β-diglyceride by way of the intermediate, cytidine diphosphate choline.

Cytidine nucleotides are also necessary for the formation of phosphatidyl ethanolamine and some of the inositol lipids.

Sphingomyelin is formed by the enzymatic transfer of phosphorylcholine from cytidine diphosphate choline to *N*-acylsphingosine (ceramide).

Evidence also is given for the conclusion that triglyceride is formed by the enzymatic esterification of D-α,β-diglyceride with a long-chain thioester of coenzyme A.

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RECENT ASPECTS OF CHOLESTEROL BIOSYNTHESIS AND CATABOLISM

Ezra Staple* and Michael W. Whitehouse
Department of Biochemistry, School of Medicine, University of Pennsylvania, Philadelphia, Pa.

The investigation of the biosynthesis of cholesterol may be divided into two general areas (see FIGURE 1): first, the pathway from acetate to the branched-chain hydrocarbon squalene and, second, the cyclization of squalene to yield the cyclic triterpenoid precursors of cholesterol and the eventual conversion of these to cholesterol. In the second area, Bloch and his collaborators have been able to make considerable progress after the initial discovery by Bloch in collaboration with Langdon[1, 2] that squalene could be converted to cholesterol in the whole animal and in liver tissue slices. This highly significant finding confirmed the hypotheses made many years before by Robinson[3] and others on purely chemical grounds and, in a sense, it unified the problem of the biosynthesis of cholesterol and that of other terpenoid substances such as rubber and the carotenoids. Tchen and Bloch[4] have also established the fact that the 30-carbon atom cyclic triterpenoid lanosterol (FIGURE 1), is an intermediate between squalene and cholesterol, and Bloch and his group have made a number of recent contributions to the complete elucidation of the mechanism of this conversion. In addition, these investigators have also learned that the enzymatic mechanism for the cyclization of squalene requires molecular oxygen, which in fact is ultimately incorporated into the three-beta-hydroxyl group of the sterol. Bucher and McGarrahan[5] found that the formation of squalene from acetate proceeds in the absence of oxygen, so that the two phases of cholesterol biosynthesis mentioned above are separated by the oxygen environment required for the respective enzyme systems.

In contrast to the considerable progress made in the second area mentioned above, the investigation of the first phase, that is, from acetate to squalene, which has been under intensive investigation for a longer time, has left a number of important and fundamental questions unanswered. The position of acetate as a precursor remains secure. The study of the pattern of its incorporation into cholesterol, which was begun by Little and Bloch,[6] has recently been brought to completion by the elegant work of Cornforth *et al.*,[7] and the results confirm those predicted on the basis of the mode of folding of the squalene chain suggested by the earlier work of Woodward and Bloch.[8] The establishment of acetoacetate as a cholesterol precursor, which appeared earlier to be assured, has not been simple. After a careful investigation of the problem, Blecher and Gurin[9] have definitely established that acetoacetate is reconverted to acetate or 2-carbon atom fragments at some stage before incorporation into cholesterol. In spite of this finding, it is still possible that acetoacetate may be incorporated as a 4-carbon atom unit, the above results indicating possibly that the rate of the reversal of the formation of acetoacetate from acetate is more rapid than the formation of cholesterol from acetoacetate.

* Established Investigator, American Heart Association, Inc., New York, N. Y.

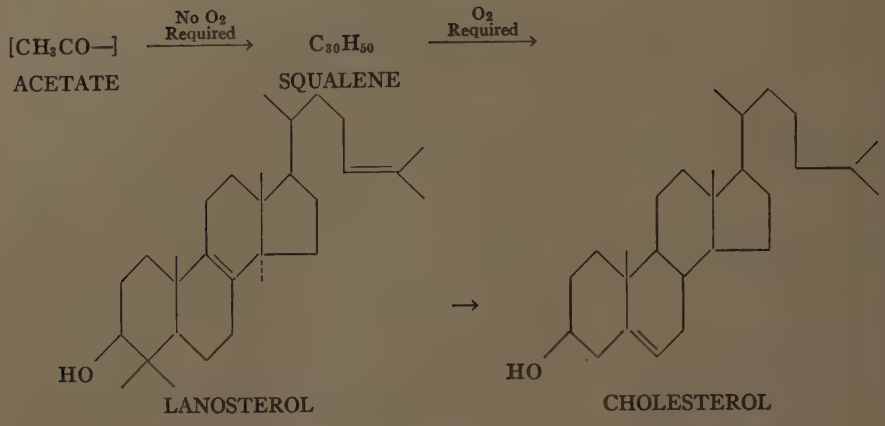


FIGURE 1

In fact, Rudney's recent report[10] also suggests that acetoacetate could be incorporated into cholesterol as a unit, since he has found that β-hydroxy, β-methylglutaric acid (HMG), a suggested cholesterol precursor mentioned below, is formed from acetoacetyl coenzyme A (CoA) and acetyl CoA in yeast. Since the discovery that acetate and acetoacetate may be incorporated into cholesterol, a large number of possible precursors have been screened. It has been reported at various times that acetone, leucine, isovaleric acid, β,β-dimethylacrylic acid (DMA), β-hydroxyisovaleric acid (HIV), and HMG have been incorporated to some extent into cholesterol. In addition, it has been found that DMA, HIV, HMG, and *trans*-β-methylglutaconic acid (MGA) are synthesized from acetate in liver tissue. In a study of the relative rates of incorporation of several of the above compounds into cholesterol, Bloch *et al.*[11] have found that DMA was converted to the greatest relative extent. The interrelationship of some of the above compounds has been suggested recently by Gurin[12] in a scheme of the general type shown in FIGURE 2. The brackets surrounding the formulas in the figure indicate hypothesized enzyme binding. However, two critical facts must be interjected here before any attempt is made to extrapolate the scheme to cholesterol biosynthesis. The first is the observation by Bloch[13] that the carbon atoms of DMA are not uniformly converted to cholesterol. It has been found that labeling in the isopropyl moiety of DMA yields a cholesterol-labeling pattern that indicates the incorporation of 6 intact DMA units, but labeling the "acetate" fragment of DMA suggests that DMA is cleaved to acetate at some stage before its incorporation into cholesterol. While this observation, as in the case of acetoacetate, may be more a reflection of the immediate metabolism of DMA itself before incorporation into the sterol, it nevertheless could speak against the idea of incorporation of the integral 5-carbon atom DMA as suggested by Bloch. Another important exception to the aforementioned scheme is the observation by Gurin and his co-workers[14] that labeled HMG, when highly purified, is found not to be a precursor of cholesterol when tested in liver homogenates and

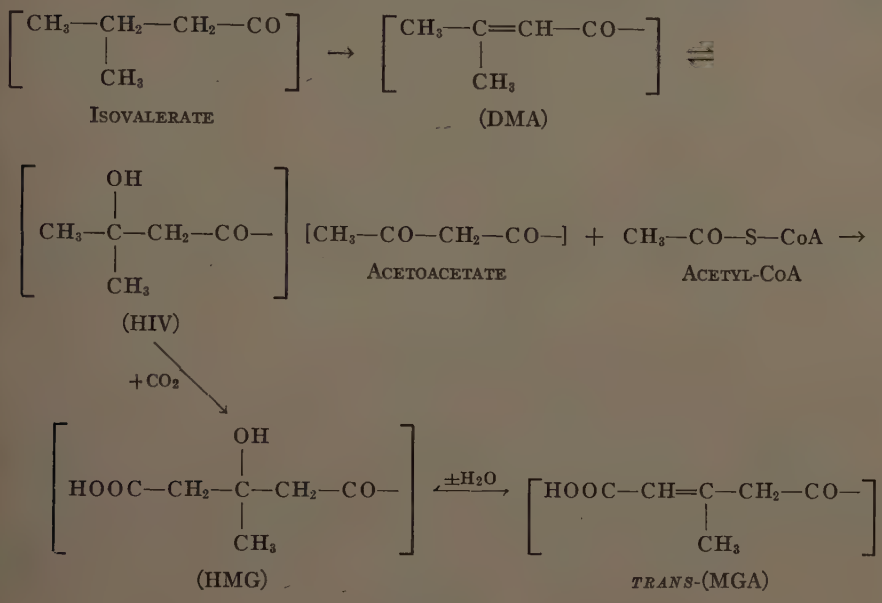


FIGURE 2

extracts. The previously reported results have been attributed to a highly active impurity formed in the reaction normally used to produce the labeled HMG. This critical point obviously needs further investigation, since it could be a minor obstacle due to the character of the enzyme preparations used or it could eliminate completely the consideration of HMG as an intermediate and relegate it to the position of a by-product of acetate metabolism in the liver preparations studied.

Probably the most significant recent finding in possible low-molecular-weight precursors of cholesterol was reported by Tavormina *et al.*[15] These investigators found that mevalonic acid (MVA), an acetate-replacing factor for bacteria, which had previously been isolated from "distillers' solubles,"[16] was incorporated almost quantitatively into cholesterol in liver extracts of the type used by Gurin and his collaborators. The chemical relationship to the previously mentioned HMG is obvious. The high rate of incorporation (the highest for any compound tested thus far) has led to extensive investigation of the mechanism by which it is converted to the sterol. It has been found that MVA is completely decarboxylated before incorporation into squalene or cholesterol, since carboxyl-labeled MVA yields practically no labeled squalene or cholesterol.[17] Experiments by Dituri *et al.*[18] and by Cornforth *et al.*[19] have demonstrated that 2-labeled MVA is incorporated into squalene, as shown in FIGURE 3. It is obvious that there has been no prior formation of an intermediate symmetrical with respect to the methyl groups, as would be expected if decarboxylation took place before incorporation to yield a "free" decarboxylated molecule, since the carbon atom adjacent to the carboxyl group appears

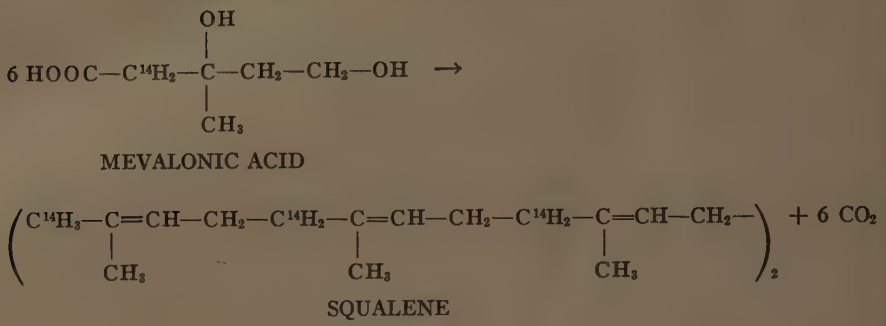
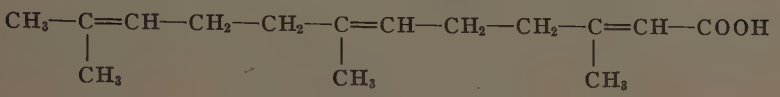


FIGURE 3

solely in the chain, while the beta methyl group appears as the branched methyl group. At present, there is no way to tell if the carboxyl group of MVA is lost before or during the condensation step, and it is possible that the MVA may preserve its initial asymmetry as a result of enzyme attachment even if the carboxyl group is cleaved before incorporation into a longer chain compound. It is necessary to inject a note of caution into all of the exciting observations and speculations that have been made with MVA; as yet, there has been no report that MVA is in fact synthetized from acetate in liver tissue. One may be dealing with a substance that, although not a direct intermediate, may be readily converted to the active biological form of the true intermediate by virtue of its particular chemical structure. The active form of precursor may still be an enzyme-bound species of "isoprene," as has been suggested. Tchen[20] has recently reported that MVA appears to be phosphorylated before incorporation into squalene in yeast preparations, and this may be a significant clue to the mechanism by which it is incorporated into squalene and ultimately into cholesterol. Obviously, much work remains to be done in this field before any real evaluation of the position of MVA on the main path of cholesterol biosynthesis can be made.

In the remaining field of study of cholesterol biosynthesis, that is, between 5- and/or 6-carbon atom precursors and squalene, there has been only one report of a compound that can be converted to cholesterol or squalene.[14] This lack enhances the possibility suggested by Bloch that the condensation of 5-carbon atom units may not proceed in a stepwise manner at all. However, the fact that 3 molecules of MVA are incorporated into squalene by 2 "head-to-tail" unions to form the 15-carbon atom farnesyl "halves" of squalene, which are in turn combined in a "head-to-head" juncture to yield the 30-carbon atom skeleton of squalene, reinforces the possibility of a 15-carbon atom precursor of the farnesol type. Recently Dituri and Gurin[21] have reported that radioactive farnesenic acid (FIGURE 4) could be isolated from squalene-synthesizing liver preparations when incubated with radioactive MVA. Possibly some compound of this type could be the sought-for 15-carbon atom intermediate. There has been some indication that cholesterol can be biosynthesized from farnesenic acid.[14]



FARNESENIC ACID

FIGURE 4

In the field of cholesterol catabolism there are two principal avenues of investigation. These follow the two main pathways for the utilization of cholesterol in mammals, the conversion of cholesterol to the steroid hormones and to bile acids.

The transformation of cholesterol to pregnenolone as a first step in the elaboration of the steroid hormones had been suspected for a long time. Results from the incubation of whole adrenal homogenates with labeled cholesterol indicated that this conversion indeed occurred.[22] The simultaneous finding of progesterone along with pregnenolone and the possibility of interconversion cast some doubt on the prior position of pregnenolone. In 1956 we reported[23] the observation that the side chain of cholesterol could be cleaved by a particle-free adrenal tissue extract to yield pregnenolone and isocaproic acid. This *in vitro* cleavage was also observed to a lesser extent in testicular and ovarian tissue extracts. The biological conversion of pregnenolone to progesterone by a 3 β-ol dehydrogenase enzyme system is well known. Hayano *et al.*[24] have outlined a scheme to indicate the formation of the principal adrenocortical steroid hormones from progesterone, so that their formation from cholesterol is now clear in principle. The formation of the sex hormones from other steroid precursors containing a larger number of carbon atoms was not too obvious until several recent observations were reported. Lynn and Brown[25] and Slaunwhite and Samuels[26] reported that progesterone could be converted to testosterone via 17-hydroxyprogesterone in cell-free testicular tissue extracts. The cleavage of the 2-carbon atom progesterone side chain to yield acetic acid was also reported by Lynn.[25] It is now clear how the androgens could be made from cholesterol, confirming previous observations of the over-all conversion of cholesterol to testosterone. It remained only to demonstrate the long-suspected convertibility of testosterone to estrone or estradiol by the removal of the angular methyl group at carbon atom 19 and the aromatization of ring A. These facts were finally established by Baggett *et al.*,[27] and the mechanism of this conversion was further elucidated by Meyer.[28] An over-all scheme for the conversion of cholesterol to the steroid hormones may be represented as in FIGURE 5. For some time the conversion of cholesterol to estrone was in doubt as a result of the experiments of Heard *et al.*,[29] who reported that administration of radioactive cholesterol to a pregnant mare did not result in the labeling of estrone. However, the conversion of cholesterol to estrone has been observed in human subjects by Werbin *et al.*[30] There is still some support among various investigators for the idea that the steroid hormones may be synthesized from acetate by some pathway independent of a route through cholesterol. Much of this is based upon finding higher specific radioactivities in the steroid metabolite than in cholesterol when both are

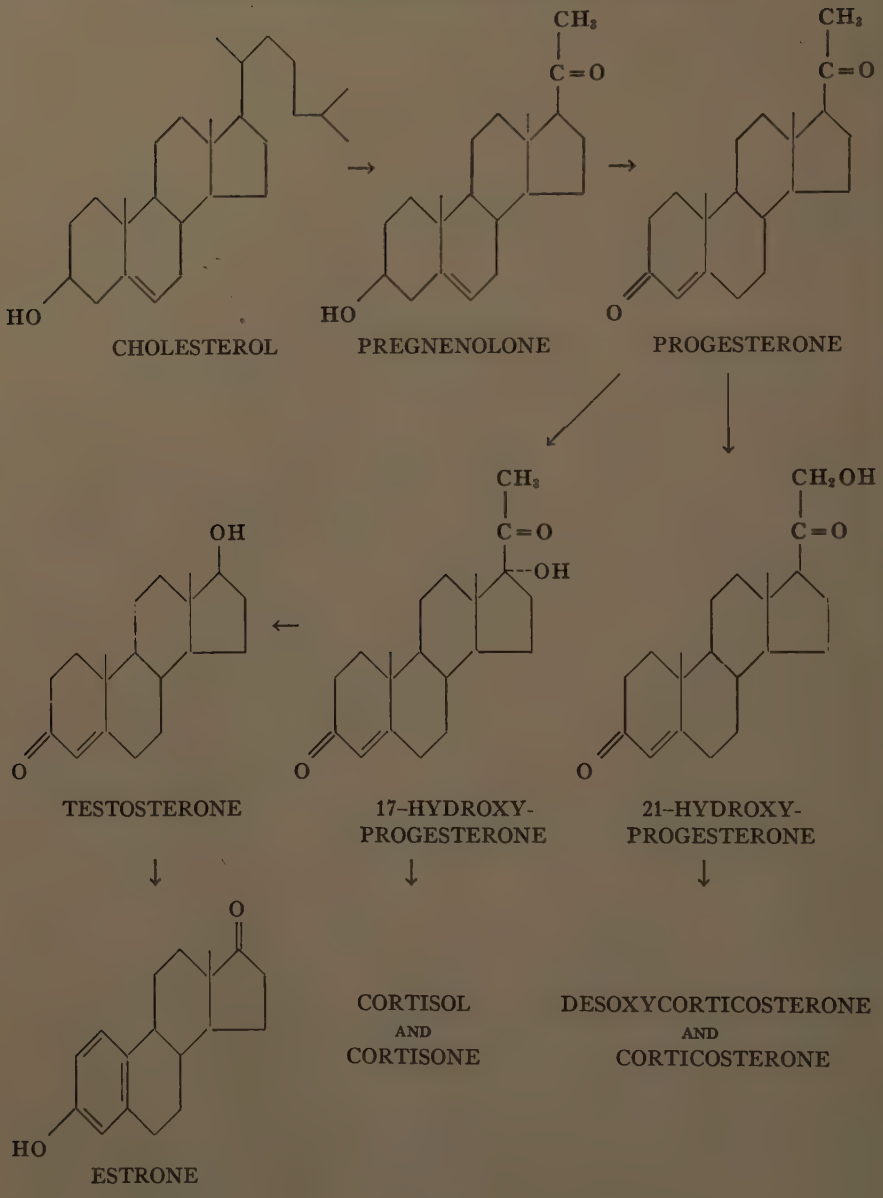


FIGURE 5

synthesized from acetate in the same tissue or tissue preparation. Of course, these discrepancies may be explained in other ways, as by the pre-existing pool size and dilution of the respective compounds to different extents. However, there still remains an element of doubt that cannot be resolved by our present

knowledge. It is therefore necessary to say at present that steroid hormones, and bile acids also, can be synthesized biologically from cholesterol, but that the obligatory role of cholesterol has not been absolutely established.

The biological conversion of cholesterol to bile acids was demonstrated

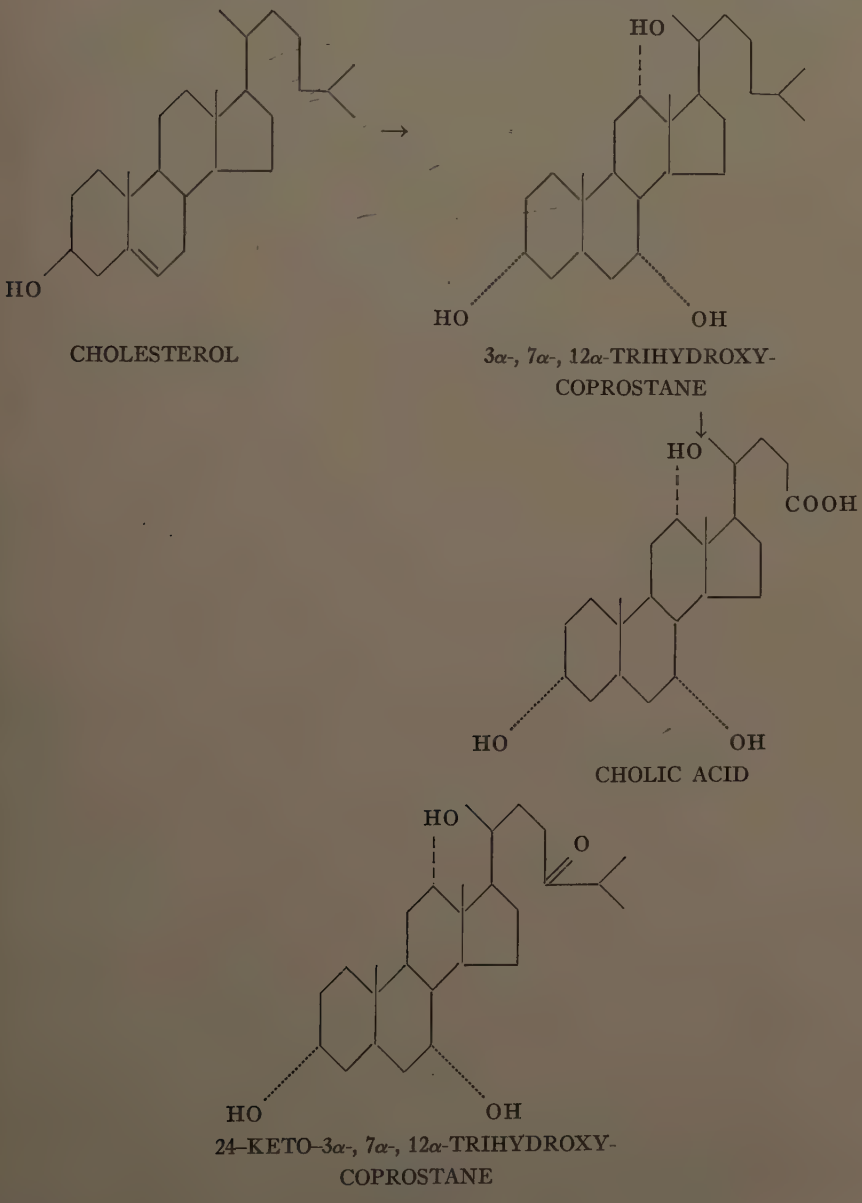
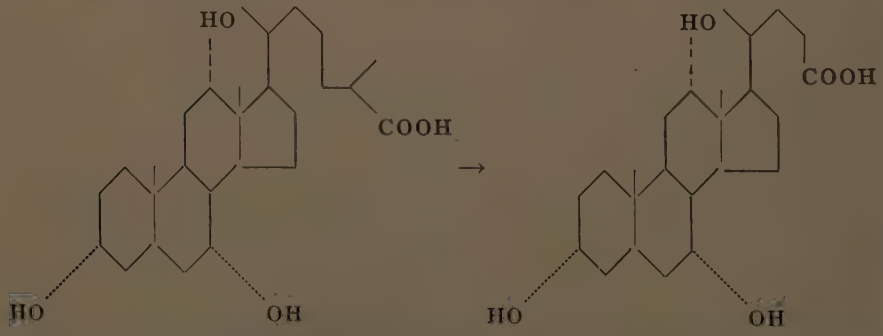


FIGURE 6

in vivo by Bloch *et al.*[31] in 1943. That this conversion involves the loss of only three terminal side-chain carbon atoms appears established by the results obtained by Zabin and Barker[32] and in our own laboratory.[33] The greatest amount of information obtained thus far on the *in vivo* transformation of cholesterol to bile acids has resulted from the painstaking investigations of Bergström and his collaborators.[34] The evidence of these workers indicates that formation of the principal bile acid, cholic acid, requires prior conversion of the cholesterol nucleus to the proper 3α-, 7α-, 12α-trihydroxy configuration before cleavage of the three terminal side-chain carbon atoms occurs (FIGURE 6). As a consequence, one would expect that 3α-, 7α-, 12α-trihydroxycoprostane (THC) would be an intermediate in cholic acid formation, and that there would be limited interconvertibility of bile acids once formed. Bergström and his group have found that THC is rapidly converted to cholic acid *in vivo*.[35] Using liver mitochondrial preparations that oxidize the terminal carbon atoms of the side chain of cholesterol to carbon dioxide, we have found that the terminal carbon atoms of THC and 24-keto THC (FIGURE 6) are also readily converted to carbon dioxide by these preparations.[36] Although quantitative comparisons are difficult to make in this type of experiment, our present evidence indicates that prior hydroxylation of the sterol nucleus considerably enhances the rate of side-chain oxidation. The detailed study of this problem *in vitro* is currently under investigation in our laboratory. In agreement with Bergström, we have come to believe that products that have an altered side chain but still possess a cholesterol nucleus, such as 25-hydroxycholesterol and 26-hydroxycholesterol, reported by Fredrickson,[37] and 25-dehydrocholesterol, reported by our laboratory[38] to be present in liver homogenates, are probably not on the direct pathway of bile acid formation and represent premature attack of the cholesterol side chain that precludes or impedes further metabolic conversion to bile acids.

Reports from our laboratory[38] and also by Fredrickson[37] have indicated that radioactive acidic substances possessing the steroid nucleus are formed from cholesterol labeled in the terminal side-chain position. Since these acids



3α-, 7α-, 12α-TRIHYDROXYCOPROSTANIC ACID

CHOLIC ACD

FIGURE 7

must have at least 26 of the original 27 carbon atoms of cholesterol and may, in fact, have the intact carbon skeleton of cholesterol, it is doubtful whether these may be considered to be mammalian bile acids which, so far as is now known, are derivatives of the 24-carbon atom cholanic acid. Steroid acids of 27-carbon atom content have been reported present in the bile of birds, reptiles, and fish.[39] The possibility that these acids may be intermediates in the formation of bile acids from cholesterol is an intriguing one. Bergström[40] has recently reported the conversion of 3α-, 7α-, 12α-trihydroxycoprostanic acid to cholic acid in rat liver homogenates (FIGURE 7).

In our studies of the oxidation of the terminal carbon atoms of the cholesterol side chain we have been using a washed liver mitochondrial preparation similar to that originally reported by Anfinsen and Horning.[41] We have found, however, that these preparations have been difficult to reproduce and that their activity has fluctuated for no readily apparent reasons. Recently, after an intensive investigation we have found a number of underlying causes for this phenomenon. Chief among these has been the too-complete removal, by washing, of dissolved protein from the medium surrounding the mitochondria. As observed in oxidative phosphorylation in mitochondria,[42] the addition of serum albumin to the medium has greatly enhanced the activity of our preparations. In fact, we have observed as much as a fortyfold increase in carbon dioxide production from the cholesterol side chain carbon atoms when supporting protein has been added to the incubation medium. It is our expectation that these findings should increase the possibilities of using such an *in vitro* enzyme preparation for studying cholesterol catabolism.

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THE MECHANISM OF CHOLESTEROL ABSORPTION*

Leon Swell, E. C. Trout, Jr., R. Hopper, Henry Field, Jr., C. R. Treadwell

Veterans Administration Center, Martinsburg, W. Va., and Department of Biochemistry, School of Medicine, The George Washington University, Washington, D. C.

In reviewing the literature directly related to sterol absorption one is impressed by the very limited area of agreement. Most investigators agree that absorbed cholesterol is transported via the lymph, that bile is obligatory for absorption, and that a major portion of the cholesterol in lymph is in the esterified form.[1-5] There is also evidence both for and against the participation of pancreatic juice and esterification in the absorption process.[6-12] There are three puzzling aspects of cholesterol absorption that cannot be explained on the basis of a direct transfer of cholesterol from the lumen of the intestine to lymph. These are: first, the appearance of fed cholesterol-4-C^{14} in lymph for periods up to several days; second, the endogenous dilution of fed cholesterol-4-C^{14} in its transfer from the lumen to the lymph and, third, the poor absorption of cholesterol-4-C^{14} when large or small doses are fed.

Recently Glover and his co-workers[13, 14] have postulated that cholesterol absorption takes place at a molecular level by way of a rapid exchange and transfer process between the lipoproteins of the cell membrane, organelles, and ground plasm. Endogenous dilution occurs due to interchange of the labeled cholesterol with inactive cholesterol on the lipoproteins. According to these workers, esterification acts in absorption only as an accelerating factor. Also, one explanation offered for the absorption of plant sterols is based on the fact that these have a certain degree of affinity for the acceptor lipoproteins that allow these sterols to participate in exchange reactions during passage across the mucosal cells. The mechanism proposed by Glover and his colleagues accounts neither for the obligatory requirement of bile nor for the appearance of labeled cholesterol in the lymph for periods up to several days after its feeding.

In previous studies[4, 5, 7-9] in which cholesterol-4-C^{14} and the lymph fistula animal have been used to study cholesterol absorption, it has been common practice to administer 1 to 3 mg. cholesterol-4-C^{14} dissolved in corn or cottonseed oil. In a fasted rat the amount of cholesterol appearing in the thoracic duct lymph during a 24-hour period is 8 to 10 mg.[15, 16] When fat alone is administered there is an increase of approximately 2 mg. in the lymph cholesterol level.[16] Thus, the administration of small amounts of cholesterol-4-C^{14} (1 to 3 mg.) dissolved in fat does not produce a chemical increase in the lymph cholesterol over that normally expected from the feeding of fat alone. However, as demonstrated by several workers, the feeding of these amounts of cholesterol-4-C^{14} is followed by the appearance of labeled cholesterol in lymph.[4, 5, 7-9] While this certainly demonstrates absorption of the labeled

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cholesterol, we suggest that such data do not necessarily describe completely the absorption of exogenous cholesterol.

In previous studies from our laboratories[17-20] several factors concerned with cholesterol absorption have been investigated: namely, pancreatic cholesterol esterase, bile, fatty acid, and sterol. The action and specificity of cholesterol esterase suggest that this enzyme is involved in the hydrolysis of dietary cholesterol esters in the lumen and the esterification of cholesterol in the mucosa during its transfer to the lymph. The absorption into the lymph of sterols other than cholesterol, such as sitosterol, dihydrocholesterol, and ergosterol, may be explained on the basis that they are esterified and compete with cholesterol for the cholesterol esterase system. Evidence was also presented that dietary fat is not essential for cholesterol absorption, since the fatty acid necessary for esterification can be derived from endogenous sources. One possible function of bile in cholesterol absorption may also be related to its obligatory requirement for cholesterol esterase activity.

In our recent studies we have determined the changes occurring in the cholesterol fractions, both by chemical and C^{14} measurements, in the lumen of the intestine, intestinal mucosa, and lymph during cholesterol absorption. Based on these findings, we propose a tentative mechanism of cholesterol absorption.

Methods and Materials

The preparation and care of the thoracic duct and thoracic-bile duct fistulae animals has been described elsewhere.[21] Twenty-four hours after the operation each animal received, by gastric intubation, 3 ml. of an aqueous emulsion containing 50 mg. blood albumin, 150 mg. glucose, and a combination of one or more of the following: cholesterol-4-C^{14}, 292 mg. oleic acid, 279 mg. sodium taurocholate, as indicated in the figures and tables that illustrate this paper. Lymph was collected for different periods of time, depending on the experiment. At various intervals the animals were sacrificed and their intestines removed. In some experiments the small intestine was sectioned into different length segments. Lipid extracts of the lymph, whole intestine, intestinal segments, and feces were made according to procedures described elsewhere.[21] Each extract was analyzed for free, ester, and total cholesterol by chemical, gravimetric, and C^{14} measurements. In order to distinguish between the amounts of cholesterol determined by radioactivity and chemical measurements, the abbreviations FC, EC, and TC will be used to designate the chemically determined free, esterified, and total cholesterol fractions; FC-C^{14}, EC-C^{14}, and TC-C^{14} indicate the corresponding fractions calculated from C^{14} activity data.

Results

Time Course of Absorption and Endogenous Dilution

The purposes of our first study were to determine how the feeding of a large dose (40 to 42 mg.) of cholesterol-4-C^{14} under physiological conditions would influence the amount of free and esterified cholesterol, as determined chem-

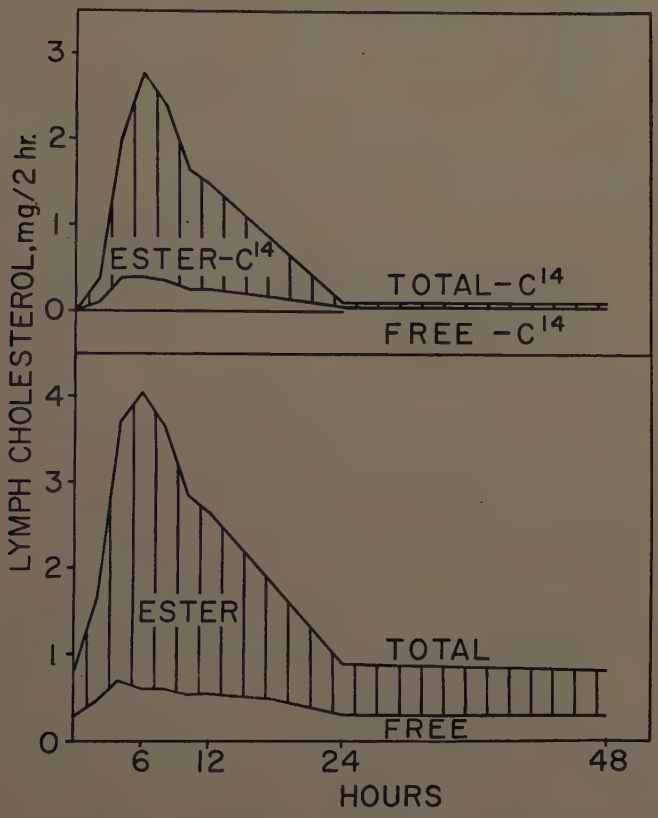

FIGURE 1. Time course of cholesterol absorption. Serial lymph samples were collected in 5 rats at 2-hour intervals following the feeding of a test meal containing 42 mg. (0.5 μc.) cholesterol-4-C^{14}, 292 mg. oleic acid, and 279 mg. sodium taurocholate. The upper curves represent the cholesterol-4-C^{14} levels; the bottom curves, the chemically determined lymph cholesterol fractions.

ically and by radioactivity in lymph, the extent of endogenous dilution, and the time course of absorption. The time-course curves of absorption are shown in FIGURE 1. During the first 2 hours very little of the fed cholesterol-4-C^{14} appeared in the lymph. Thereafter, the amounts of TC and TC-C^{14} increased rapidly, reaching a peak in 6 hours. During the height of absorption the EC and EC-C^{14} were 85 and 86 per cent, respectively. In several animals the EC-C^{14} at that time was 90 per cent. In contrast, the FC and FC-C^{14} increased very little during the period of rapid absorption. These results indicate that there is a definite relationship between the degree of esterification and the rate of cholesterol absorption. These findings are not in agreement with those of Chaikoff and his co-workers,[9] who reported that, regardless of the amount of the fed cholesterol recovered in lymph, about 70 per cent of that cholesterol was esterified.

FIGURE 2 shows the cumulative percentage of the fed cholesterol-4-C^{14} that

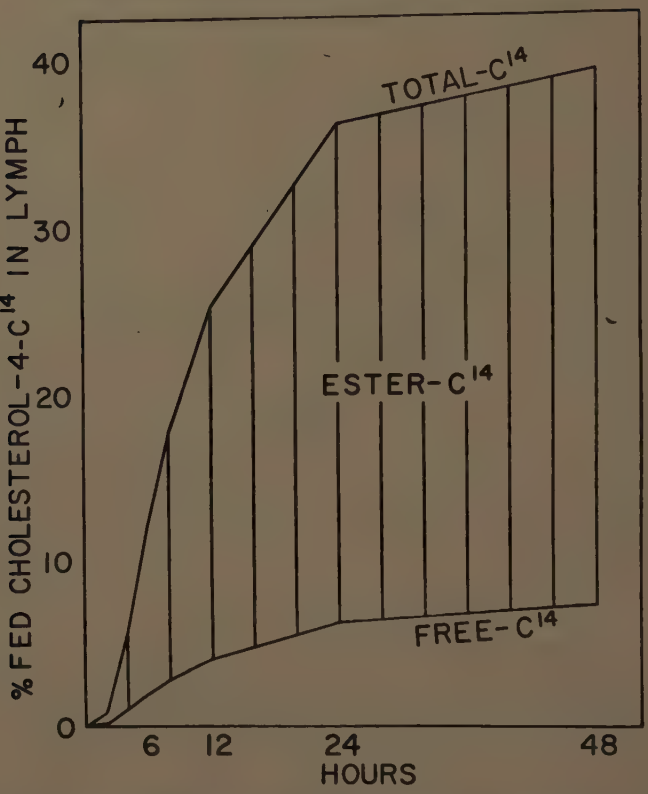

FIGURE 2. The cumulative percentage of fed cholesterol-4-C^{14} recovered in lymph over a 48-hour period. The composition of the test meal is the same as in FIGURE 1 (five rats in the group).

was recovered in the lymph over a 48-hour period. At the end of 12 hours 25 per cent of the fed cholesterol-4-C^{14} appeared in the lymph. Although the lymph resumed its clear postabsorptive state after 12 hours, an additional 14 per cent was recovered during the next 36 hours.

FIGURE 3 shows the specific activity of lymph cholesterol at various intervals during absorption. The specific activity of the free and esterified cholesterol in lymph increased and then decreased in parallel with the changes in the levels of the cholesterol fractions shown in FIGURE 1. The specific activity of the esterified cholesterol during the first 2 hours was 1400 cpm/mg., indicating considerable endogenous dilution. During the height of absorption, at 6 hours, the specific activity of this fraction increased to 4200 cpm/mg., which still represented a 30 per cent endogenous dilution of the fed cholesterol-4-C^{14}. While the specific activity of the free cholesterol followed the same pattern as the esterified cholesterol, it had a lower specific activity at the various time intervals. These results confirm and extend the earlier findings of Biggs and his co-workers[3] of considerable endogenous dilution of fed cholesterol-4-C^{14} in its transfer from the lumen to the lymph.

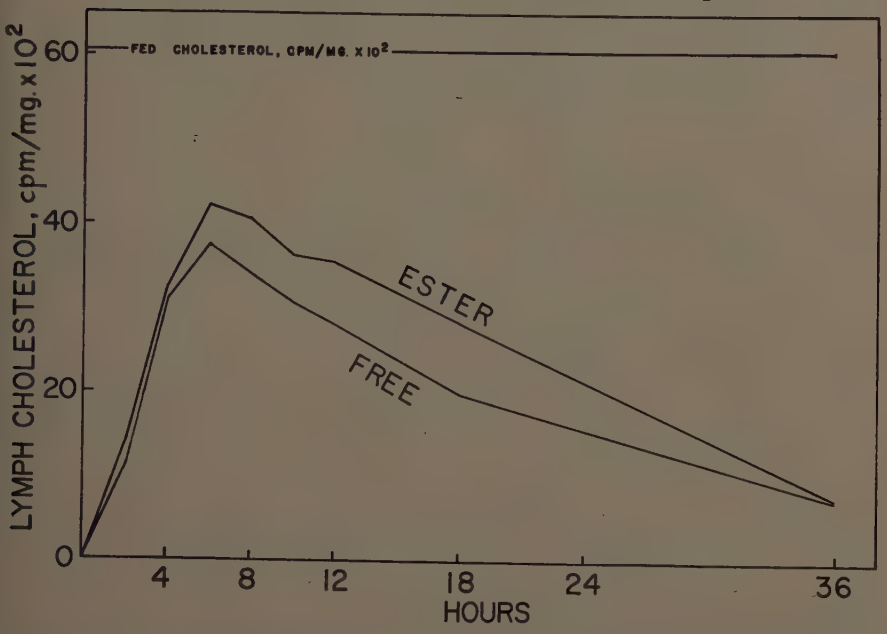

FIGURE 3. The specific activity of free and esterified lymph cholesterol at different time intervals during cholesterol absorption. The composition of the test meal is the same as in FIGURE 1 (five rats in the group).

To determine the location of this endogenous dilution, the specific activity of the cholesterol in the test meal, the contents of the lumen and lymph were compared after a 6-hour feeding period; these results are shown in FIGURE 4. The specific activity of the test meal was 4900 cpm/mg. The specific activity of the total cholesterol present in the lumen of the intestine was 4400 cpm/mg., demonstrating that very little endogenous dilution of the fed cholesterol occurred in this area. The specific activity of the total cholesterol in lymph was 2400 cpm/mg., which represents a 46 per cent endogenous dilution of the fed cholesterol-4-C^{14} in the lumen. This marked dilution in specific activity of the fed cholesterol-4-C^{14} from the lumen to the lymph must have occurred by mixing with a pool of endogenous cholesterol in the intestinal mucosa. The amount of esterified cholesterol in the intestinal wall is not adequate (1 mg.) to account for this degree of endogenous dilution. Also, the appearance of labeled cholesterol in the lymph for periods up to several days[3, 7] strongly suggests a dynamic pool several orders of magnitude greater than can be accounted for by the esterified cholesterol fraction in the mucosa. There is adequate free cholesterol in the intestinal wall to account for this endogenous dilution.

Free and Esterified Cholesterol Pools of Mucosa

Feeding of large amounts of cholesterol-4-C^{14}. In order to characterize the cholesterol pools of mucosa, the changes occurring in the cholesterol levels (both by chemical and C^{14}-activity measurements) of the intestine and lymph during various periods of cholesterol absorption were deterimined; the results

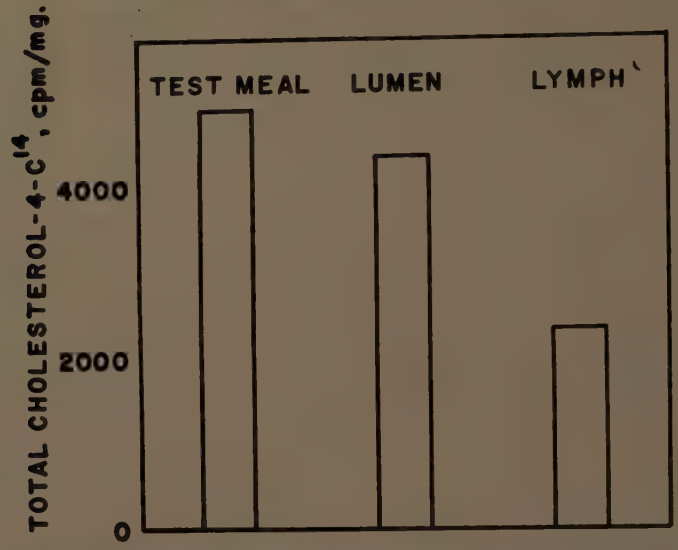

FIGURE 4. Endogenous dilution of fed cholesterol-4-C^{14} in the lymph and lumen of the intestine. Values are the average of 4 rats sacrificed 6 hours after a test meal containing 40 mg. (0.5 μc.) cholesterol-4-C^{14} (4900 cpm/mg.), 292 mg. oleic acid, and 279 mg. sodium taurocholate.

TABLE 1

CHOLESTEROL-4-C^{14} OF LYMPH AND INTESTINE AT VARIOUS TIME INTERVALS FOLLOWING THE FEEDING OF CHOLESTEROL-4-C^{14}

Time* (hours)	Specific activity total cholesterol		Administered cholesterol-4-C^{14} recovered	
	Lymph	Intestine†	Lymph	Intestine†
	cpm/mg.	*cpm/mg.*	*per cent*	*per cent*
6	2443	957	8.9	10.3
24	1822	747	20.5	7.3
48	1632	158	27.3	1.5

* Collection time for lymph and time of sacrifice after administration of test meal containing 40 mg. (0.5 μc.) cholesterol-4-C^{14} (4900 cpm/mg.), 292 mg. oleic acid, and 279 mg. sodium taurocholate; 3 to 4 rats per group.

† Represents whole intestine, both small and large.

are shown in TABLE 1. During a 6-hour period 8.9 per cent of the fed cholesterol-4-C^{14} was recovered in the lymph. At the end of 6 hours there was 10.3 per cent of the fed cholesterol-4-C^{14} in the intestinal wall, which was somewhat greater than the amount appearing in lymph during this period. After 24 hours the intestinal wall still contained considerable amounts of cholesterol-4-C^{14} (7.3 per cent) and, at 48 hours, there remained 1.5 per cent of the fed dose. The decline in the percentage of cholesterol-4-C^{14} from 24 to 48 hours in the intestine was approximately equal to the quantity transferred to the lymph during this same period. The specific activity data on the intestine indicate

TABLE 2

CHOLESTEROL AND CHOLESTEROL-4-C^{14} OF LYMPH AND INTESTINAL SEGMENTS FOLLOWING THE FEEDING OF CHOLESTEROL-4-C^{14}

Tissue*	FC	FC-C^{14}	EC	EC-C^{14}
	mg.	*mg.*	*mg.*	*mg.*
Lymph	1.6	0.6	6.1	3.1
Small intestine	14.4	4.3	2.5	1.4

	Specific activity		Administered cholesterol-4-C^{14} recovered
	Free	Ester	
	cpm/mg.	*cpm/mg.*	*per cent*
Lymph	2311	3022	8.8
Int. seg. 1	1571	3170	1.9
Int. seg. 2	2049	3538	6.2
Int. seg. 3	1966	3186	4.5
Int. seg. 4	965	806	1.0

* The values are the average for 5 rats sacrificed 6 hours after administration of test meal containing 42 mg. (0.5 μc.) cholesterol-4-C^{14} (6000 cpm/mg.), 292 mg. oleic acid, and 279 mg. sodium taurocholate. The small intestine was divided into segments of 6, 12, 12, and 6 inches, numbered respectively, 1, 2, 3, and 4 from the upper duodenal end.

that considerable endogenous dilution of the fed cholesterol-4-C^{14} occurred in the intestinal wall. The marked differences between the specific activity of the total cholesterol of lymph and intestine appeared to be due to dilution by the cholesterol of the large intestine and perhaps other areas of the small intestine not taking part in cholesterol absorption.

As a result of these findings it became of interest to examine the small intestine more closely to locate the area most active in absorption. Accordingly, the small intestine was sectioned into 4 segments, as indicated in TABLE 2. While each of the segments had appreciable quantities of labeled cholesterol, segment 2 had the highest level, which indicates that this area is most active in cholesterol absorption. The specific activity relationships between the intestine and lymph demonstrate more clearly that the FC and EC of the lymph are derived from the free cholesterol of the mucosa. Segment 2 had specific activity values for free and esterified cholesterol very close to those of lymph. These relationships also show that, while the esterified cholesterol is transferred from the mucosa to the lymph without appreciable dilution, the free fractions in both are still diluted with cholesterol not involved in absorption.

The small intestine of a fasting lymph fistula rat contains approximately 1 mg. of esterified cholesterol. The feeding of 42 mg. cholesterol-4-C^{14} (TABLE 2) was followed by an increase in the EC of the small intestine (2.5 mg.). During the height of cholesterol absorption as much as 31 per cent of the TC-C^{14} was present as EC-C^{14} for segment 2. These results are contrary to those of Favarger and Metzger,[6] who reported that there is no increase in the amount of EC and that the EC does not become labeled during the absorption of cholesterol unless the animals have previously received a diet high in choles-

TABLE 3

LYMPH AND INTESTINAL CHOLESTEROL FOLLOWING THE FEEDING OF A SMALL AMOUNT OF CHOLESTEROL-4-C^{14}

Emulsion* additions	FC	FC-C^{14}	EC	EC-C^{14}	Specific activity		Administered cholesterol-4-C^{14} recovered
					Free	Ester	
Lymph							
	mg.	*mg.*	*mg.*	*mg.*	*cpm/mg.*	*cpm/mg.*	*per cent*
3.3 mg. cholesterol-4-C^{14}	0.74	0.02	1.55	0.08	2842	5292	3.1
3.3 mg. cholesterol-4-C^{14}, 292 mg. oleic acid, 279 mg. s. taurocholate	1.50	0.09	3.00	0.30	5771	9984	11.9
Small intestine†							
3.3 mg. cholesterol-4-C^{14}	12.55	0.34	1.24	0.04	4501	5096	11.7
3.3 mg. cholesterol-4-C^{14}, 292 mg. oleic acid, 279 mg. s. taurocholate	13.05	0.58	1.24	0.09	6427	9414	20.5

* Five animals per group sacrificed at 6 hours after test meal; specific activity fed cholesterol-4-C^{14}, 1×10^6 cpm/mg.

† Specific activity values on the small intestine are for segment 2 (TABLE 2).

terol. Comparison of the FC of the small intestine with that of a control group (12.1 mg.) showed that there was an increase in this fraction of 2.3 mg.

Feeding of small amounts of cholesterol-4-C^{14}. The feeding of 40 to 42 mg. cholesterol-4-C^{14} led to chemical increases in lymph and intestinal cholesterol and the labeling of the cholesterol fractions in both sites. Since the feeding of small amounts of cholesterol-4-C^{14} (1 to 3 mg.) dissolved in fat does not produce a chemical increase in lymph cholesterol over that expected from feeding fat alone, it appeared that the feeding of these small amounts should not produce an increase in the size and turnover rate of the cholesterol fraction in the mucosa. We have found that, following the feeding of 3.3 mg. cholesterol-4-C^{14} alone (TABLE 3), there is no increase in the lymph cholesterol when compared to a fasting control group (TABLE 4). The specific activity of the esterified fraction of lymph was higher than the specific activity of the free fraction. These differences can be accounted for by greater dilution of the free cholesterol fraction by cholesterol from extraintestinal sources than would occur when larger amounts of cholesterol-4-C^{14} are fed.

Table 3 also shows the effects of adding sodium taurocholate and oleic acid to the test meal. These produced a greater transfer of cholesterol-4-C^{14} to the lymph than was observed when cholesterol-4-C^{14} was fed alone. Also, the specific activity of both the free and esterified fractions in lymph was considerably higher than in the previous group. Comparison of the cholesterol levels in the lymph of both groups shows that the addition of oleic acid and sodium taurocholate doubled the lymph FC and EC. These differences can not be accounted for on the basis of the cholesterol-4-C^{14} present in the lymph. The appearance of the additional unlabeled cholesterol must be related to

TABLE 4

CHOLESTEROL OF LYMPH AND INTESTINE OF THE FASTING LYMPH FISTULA RAT

FC	EC	TC	EC/TC
Lymph*			
mg. 0.8	*mg.* 1.4	*mg.* 2.2	*per cent* 63.6
Small intestine*			
12.1	1.0	13.1	7.7

* The values are the average for 5 animals fasted for 30 hours after the operation. Lymph was collected from hours 24 through 30, and the animals were sacrificed and the small intestine removed.

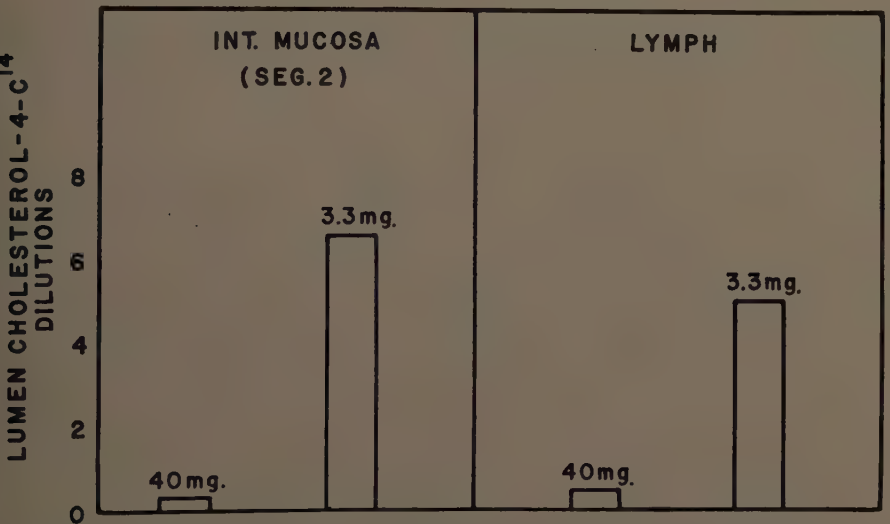

FIGURE 5. Endogenous dilution of small and large amounts of fed cholesterol-4-C^{14}. There were 4 animals per group; each animal of the first group received 40 mg. (0.5 μc.) cholesterol-4-C^{14}, 292 mg. oleic acid, and 279 mg. sodium taurocholate; the second group received the same emulsion with 3.3 mg. (0.5 μc.) cholesterol-4-C^{14}. The time of sacrifice was 6 hours.

processes involved in fat absorption; this would lead to increased turnover of the free cholesterol pool of mucosa. The chemical data (TABLE 3) on the intestine demonstrate that there was no increase in either FC or EC fractions when compared to the control group (TABLE 4).

If fed cholesterol-4-C^{14} during passage through the mucosa is mixed with a pool of free cholesterol, it would be predicted that the dilution of the fed cholesterol-4-C^{14} by the pool would be greater for a small dose than for a large one. Our data (FIGURE 5) show that when a small dose of cholesterol-4-C^{14}

is fed with sodium taurocholate and oleic acid it is diluted fifteen- to twentyfold more in the intestine than is the large dose.

Pool size. On the assumption that the esterified cholesterol of the intestine was derived from the free fraction and that dilution of the esterified cholesterol of segment 2 was minimal, the intestinal pool was calculated to be approximately 8.5 mg. for the 6-hour absorption period when 40 to 42 mg. of cholesterol-4-C^{14} was fed. When 3.3 mg. of cholesterol-4-C^{14} was fed, the intestinal pool was found to be 5.5 mg. These findings support the view that the free cholesterol pool of mucosa expands when large amounts are fed, and that an increased turnover and transfer to the lymph results. The size of intestinal pool in the fasting animal would be approximately 5 to 6 mg., and this would indicate that the pool turns over once every 24 hours during fasting.

Bile Salts and the Cholesterol Pool of Mucosa

It became of interest to determine what role bile salts might have in the entrance of cholesterol-4-C^{14} into the free cholesterol pool of mucosa and its subsequent transfer to the lymph. Accordingly, rats with thoracic and bile duct fistulae were given various test meals, as indicated in FIGURE 6. A small

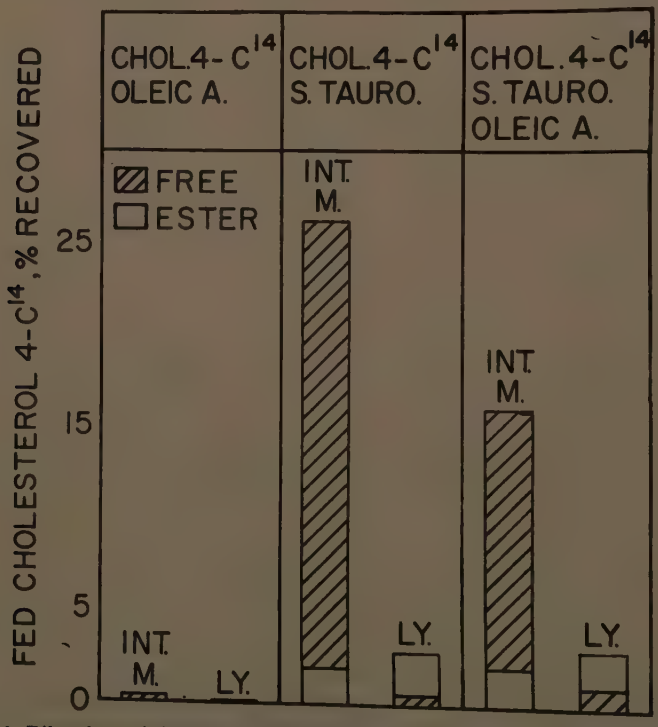

FIGURE 6. Bile salts and the cholesterol pool of mucosa. There were 3 groups of 5 thoracic-bile duct fistulae animals. Group 1 received 3 mg. (0.5 μc.) cholesterol-4-C^{14}; in addition to the cholesterol-4-C^{14}, groups 2 and 3 received 279 mg. sodium taurocholate and 292 mg. oleic acid plus 279 mg. sodium taurocholate, respectively. The time of sacrifice was 6 hours.

amount of cholesterol-4-C^{14} (3 mg.) was fed so that we might study the effect of bile salts on the transfer of endogenous cholesterol to the lymph. With oleic acid in the test meal there was virtually no uptake of cholesterol-4-C^{14} by the intestinal wall and no transfer to the lymph. The addition of sodium taurocholate alone to the test meal was followed by uptake of cholesterol-4-C^{14} by the intestinal wall and transfer to the lymph. As in the previous experiments, most of the TC-C^{14} of the intestine was present as FC-C^{14}, with the reverse true in lymph. The addition of oleic acid and taurocholate to the test meal was also followed by uptake of cholesterol-4-C^{14} by the intestinal wall and transfer to the lymph, but this uptake was less than when oleic acid was omitted from the emulsion. While there was transfer of cholesterol-4-C^{14} to the lymph when bile salts were added to the test meal, it was less than was previously observed in the animals with their bile supply intact. This demonstrates the importance of enterohepatic circulation of bile salts in cholesterol absorption. The results of this experiment provide evidence that bile salts are necessary for the entrance of cholesterol into the mucosa. However, the data do not eliminate the possibility that bile salts may also function in later steps of cholesterol absorption. The present data suggest that a complex of cholesterol and bile salts is formed in the lumen and that this complex then enters the intestinal wall. Dietary fat and emulsification may not be essential for this process.

Discussion

Based on the findings of the present and previous studies,[1, 3, 4, 8, 15-24] we propose the following tentative scheme for the mechanism of cholesterol absorption (figure 7). Free and esterified dietary cholesterol and triglyceride

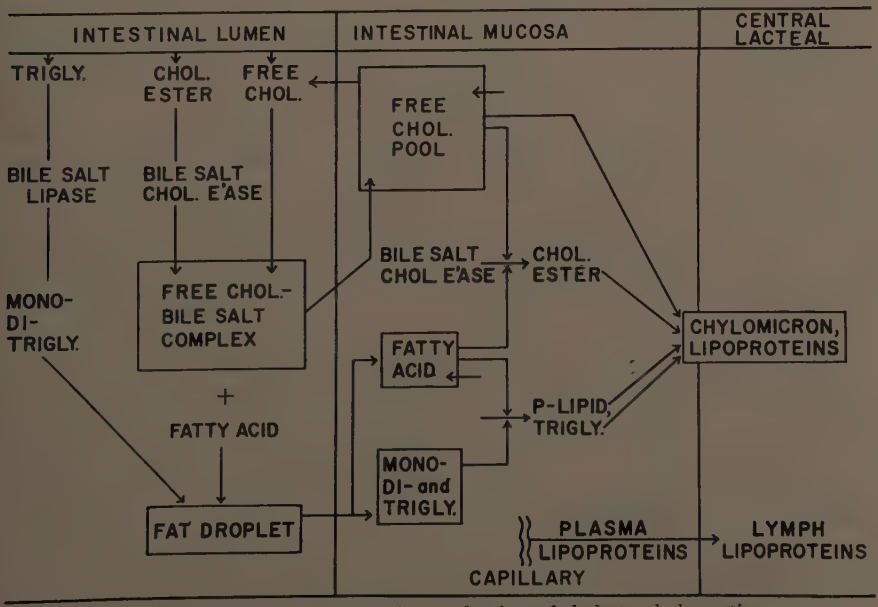

Figure 7. Tentative scheme for mechanism of cholesterol absorption.

enter the lumen of the small intestine. These substances are mixed with cholesterol, other lipids of endogenous origin, bile, and pancreatic secretions. The cholesterol esters and triglycerides are hydrolyzed. The free cholesterol, perhaps as a complex with bile salts, along with fatty acid, pancreatic cholesterol esterase, and the products of triglyceride digestion, pass into the intestinal mucosa. The free cholesterol becomes mixed with the pool of free cholesterol in the mucosa. This pool is constantly being turned over by the synthesis of cholesterol in the mucosa,[25] followed by its esterification and passage with a small amount of free cholesterol into the lacteals. That this occurs is shown by the constant amount of cholesterol (8 to 10 mg.) appearing in the lymph of a fasting animal during a 24-hour period[16] and the labeling of this cholesterol when a tracer dose is given. Also, when bile is diverted from the small intestine, the total lymph cholesterol drops to 2 to 3 mg. and, of this amount, only one-third is present as esterified cholesterol.[26] When 40 to 42 mg. of cholesterol-4-C^{14} are fed, the entrance of free cholesterol from the lumen tends to increase the pool size and, at the same time, conditions are favorable for the esterification of cholesterol due to the presence of bile salts and cholesterol esterase. There then results an increased synthesis of cholesterol esters, which pass into the central lacteal. Since very little cholesterol ester is found in the mucosa at any one time, it may be presumed that the esterification reaction takes place at the site of transfer of cholesterol to the central lacteal. The cholesterol esters do not pass into the lymph alone, but are transferred along with the triglycerides, phospholipids, free cholesterol, and protein that constitute lipoproteins and chylomicrons. Free cholesterol is probably an essential part of the chylomicron structure and is drawn from the free cholesterol pool for this purpose. Consistent with these views are the observations that during cholesterol absorption the phospholipid and neutral fat content of lymph increase[16, 24] and that during fat absorption there is an increase in lymph cholesterol.[15, 16, 27] If this mechanism of cholesterol absorption is correct, the appearance of FC-C^{14} in lymph is a necessary part of the transport system for the esterified cholesterol. It further follows that cholesterol absorption is closely interrelated with the absorption and transport of other lipids. When large amounts of cholesterol are fed, an increased synthesis of triglyceride and phospholipid must occur in the mucosa to support the increased formation of chylomicrons; conversely, during fat absorption or when a small amount of cholesterol is fed with fat, there must be an increased synthesis of cholesterol by the mucosa.

Finally, the appearance of additional cholesterol in the lymph over that which would be present during fasting after feeding cholesterol is due to increases in the rate of the processes going on at all times in the mucosa. The appearance of this additional cholesterol may be looked upon as the result of a homeostatic mechanism for maintaining the constancy of the cholesterol fractions of the intestinal mucosa.

Summary

The present studies have provided evidence that a metabolic pool of free cholesterol exists in the intestinal mucosa with which cholesterol from the

lumen of the intestine is mixed prior to its esterification and transfer to the lymph. The existence and turnover of such a pool has made it possible to explain certain aspects of cholesterol absorption and to propose a tentative mechanism of cholesterol absorption.

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STRUCTURE AND HOMOGENEITY OF THE LOW-DENSITY SERUM LIPOPROTEINS*

Frank T. Lindgren, Alex V. Nichols, Thomas L. Hayes,
Norman K. Freeman, John W. Gofman

Donner Laboratory of Medical Physics, Division of Medical Physics, Department of Physics, and the Radiation Laboratory, University of California, Berkeley, Calif.

Introduction

Although human serum lipoproteins have been studied intensively over the past decade, we still lack complete information concerning the chemical composition and structure of these macromolecules. One difficulty with regard to such studies is the wide range of lipoprotein classes that exist as part of the total blood lipoprotein spectra. It might be estimated, for instance, that in a typical serum lipoprotein distribution (as shown schematically in FIGURE 1 for a normal 45-year-old male) there might easily be the order of 100 lipoprotein classes, each of which could be distinguished from the others by physical or chemical means or by some combination of analytical methodologies now available.

In this presentation we shall examine variations in composition that occur within each broad lipoprotein group and consider further the evidence for homogeneity of lipoproteins isolated within one narrow S_f band.

There is evidence that the larger low-density lipoproteins, particularly those above S_f 100, may be lipoprotein complexes of limited stability. Furthermore, these macromolecular units present in the blood stream may be in a state of constant transformation, possibly reflecting a relationship to the breakdown and transport of all classes of lipids within the blood compartment. Considering this, we shall present evidence bearing on a hypothesis of a lipoprotein complex model for these very large low-density lipoproteins.

Methods

Lipid analyses were performed on extracts obtained from isolated lipoproteins by the method of Sperry and Brand.[1] In the broad lipoprotein band composition studies of fasting sera, silicic acid chromatography was combined with infrared spectrophotometric analysis.[2] In the narrow-band S_f 6 to 8 study a modified procedure of total lipid analysis by infrared absorption[3] was combined with chemically determined free and total cholesterol.[4]

All ultracentrifugal preparative work was carried out using a 40.3 Spinco rotor in a Spinco Model L ultracentrifuge equipped with a diffusion pump. Rotor temperature was maintained at approximately 18° C. during each of the preparative runs. The broad-band lipoprotein isolations were made as described elsewhere.[5] However, a sedimentation equilibrium system was set up for the isolation of the S_f 6 to 8 narrow-band lipoproteins. To serum was added solid anhydrous NaBr or an appropriate volume of dense NaBr solution

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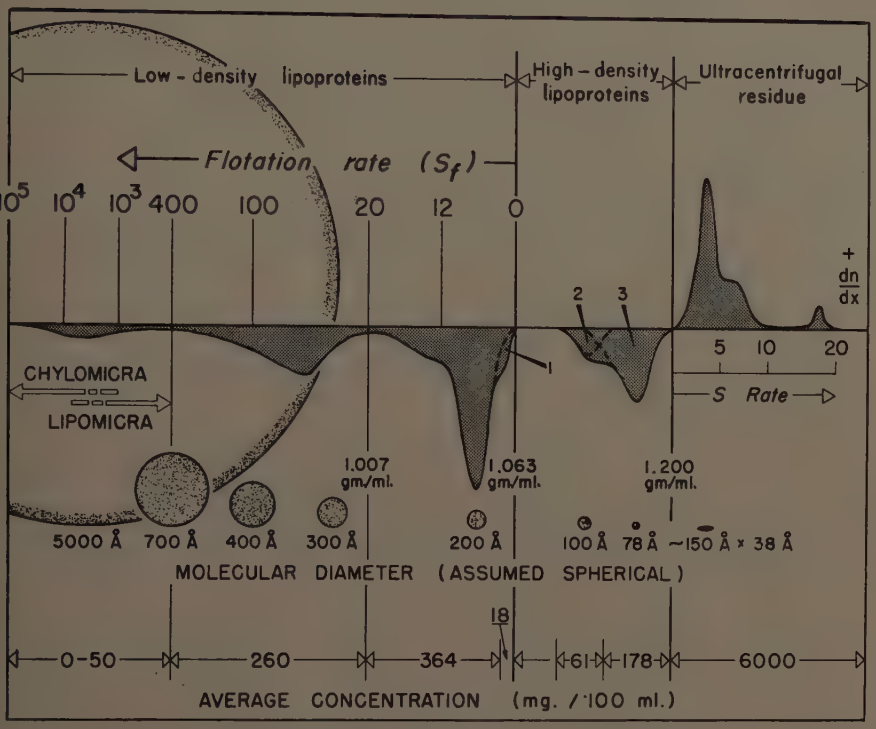

FIGURE 1. The ultracentrifugal composition of human serum, showing relative molecular sizes. Serum concentrations indicated are the average for 45-year-old males. The ultracentrifugal residue (or subnatant from the 1.20-gm./ml. preparative run) is plotted on thirty-fold-reduced dn/dx scale and contains the total ultracentrifugal albumin, globulin, and "20" components. These components have sedimentation rates of approximately 4, 7, and 19 Svedbergs, respectively.

such that the small molecule solution density of the mixture was 1.032 gm./ml. Three ml. of this solution was placed in a 6-ml. preparative tube, and beneath it 3 ml. of a similar NaBr serum mixture of density* 1.038 gm./ml. was layered by means of a syringe and spinal needle. Ultracentrifugation was carried out at 40,000 rpm for 48 hours. Both the serum salts and the added NaBr contributed to the net sedimentation equilibrium salt distribution. For the Spinco 40.3 rotor operating at 40,000 rpm the approximate equilibrium salt distribution for a NaBr solution alone from the top milliliter to the bottom milliliter of the preparative tube is such that the continuous density gradient formed is approximately 88 per cent of the initial mean salt concentration at the top and approximately 114 per cent of the initial concentration at the bottom. However, for serum mixtures, the (equilibrium) concentration shifts of the serum salts (such as NaCl) are only about one half as much as that for NaBr, hence their partial contribution to the salt gradient is less. On the other hand, CsCl (a salt of much higher molecular weight) will provide an equilibrium

* Unless otherwise stated, all densities refer to 20° C.

gradient under comparable circumstances of about twice that provided by NaBr. The advantage of using NaBr is that it is inexpensive and, in contrast to a similar salt such as KBr, it is more soluble (up to 48.5 per cent by weight, corresponding to a density of 1.534 gm./ml.). Further, in lipoprotein studies using only one salt (such as NaBr) densities may be conveniently determined indirectly by simple and accurate refractive index measurements. In this study all density measurements were made with a Bausch and Lomb precision refractometer with appropriate calibrations by pycnometry.

Physical Properties of Lipoproteins

Although physical size might be considered the basic and unique feature of the serum lipoproteins, the physical property of hydrated density can intrinsically provide more information about any particular lipoprotein under consideration. It suggests, for instance, how the lipoprotein may be isolated (if more dense than 1.006 gm./ml.) by 2 stages of differential-density preparative ultracentrifugation or by a single stage of isolation on a sedimentation-equilibrium salt gradient (as described under *Methods*). Furthermore, knowing the hydrated density of a lipoprotein allows estimation of the relative content of protein and lipid. FIGURE 2 shows the closely linear relationship that exists between lipoprotein protein content and estimated hydrated density.[6,7] This relationship would be anticipated, since most of the lipids composing serum

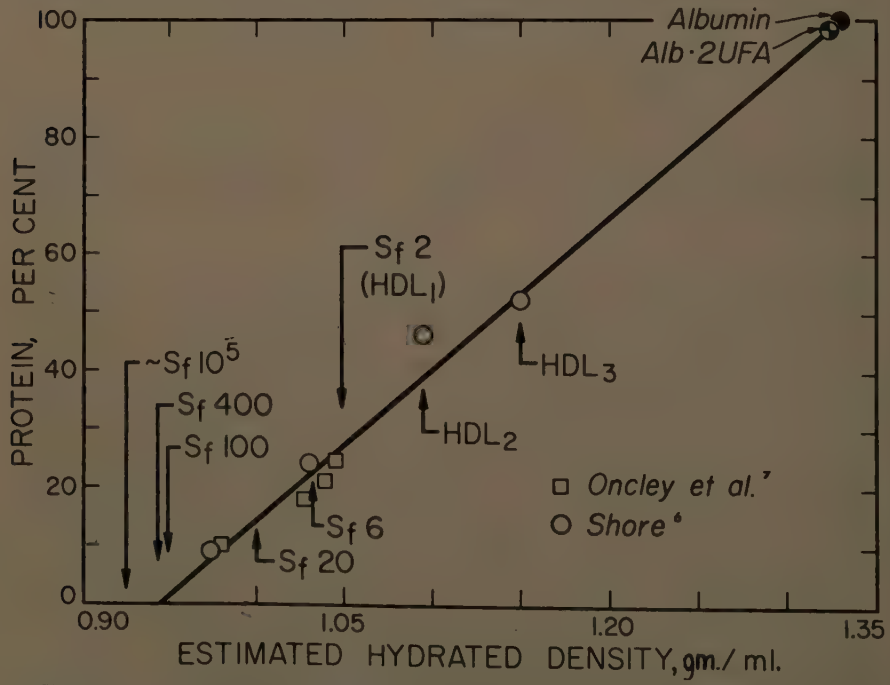

FIGURE 2. The relationship between per cent protein content and estimated hydrated density.

lipoproteins have densities in the neighborhood of 0.9 to 1.0 gm./ml. and in the assembly of a lipoprotein, volume additivity of both lipid and protein components might be expected to hold as a first approximation.

The physical property of size is also a characteristic and distinguishing feature of lipoproteins. In the low-density spectrum (σ's < 1.05 gm./ml.) there are many examples from both normal and pathological individuals that exhibit an almost continuous spectrum of lipoproteins ranging in size from the smallest 200-Å lipoproteins within the S_f 0 to 10 class to the largest chylomicra ($\sim$10,000 Å). In the high-density lipoprotein spectrum, which extends from about 1.08 to 1.17 gm./ml. in hydrated density,[8] there exists a series of lipoproteins the molecular weights of which range from approximately 150,000 to 400,000. FIGURE 1 presents the known spectra of serum lipoproteins, together with illustrations of their relative sizes, assuming spherical molecules, as calculated from ultracentrifugal data using Stokes' frictional factor for spheres.

Taken together, these lipoprotein molecules account for approximately 90 to 95 per cent of all the circulating serum lipids. Additional lipids may be present in the form of lipoproteins more dense than 1.20 gm./ml. that are as yet uncharacterized.[9-12] Also, as is also well known from the work of many contributors, including Teresi and Luck,[13] Gordon,[14] Dole,[15] and Goodman,[16] to mention only a few, important and specific fatty acid complexes with serum albumin exist at low concentrations in serum.

Chemical Properties of the Serum Lipoproteins

Protein content. As already mentioned, on a physical basis the total protein content of the lipoproteins is related intimately to the physical property of hydrated density of the lipoproteins. Furthermore, there is a general trend to increasing size with decreasing percentage of protein content that holds over the entire lipoprotein spectra. The character of this protein moiety is crucial to our understanding of the structure of each class of lipoproteins, particularly to the very large low-density lipoproteins. As a broad feature, the amino acid compositions of each of the lipoprotein classes of serum appear to be similar to one another but different from the amino acid compositions of such blood protein as albumin, fibrinogen, and gamma-globulin.[17] However, some differences in amino acid composition between low- and high-density lipoproteins have been reported.[18]

Of more specific characterization are the N- and C-terminal amino acids of the protein units present as part of the lipoproteins. The N-terminal amino acid composition of the major lipoprotein classes significantly differs one from another, whereas each class appears to be characterized by a relatively distinct but complicated protein moiety containing more than one N-terminal amino acid[18-20] (FIGURE 13). This relatively complicated picture is particularly evident from analysis within the S_f 0 to 20, S_f 20 to 100, and S_f100 to 10^5 regions of the low-density lipoprotein spectrum.[21]

Broad-band lipid composition of all major lipoprotein groups. FIGURE 3 shows the average lipid composition of the 3 broad lipoprotein classes obtained from 9 fasting adult individuals, 4 of whom were clinically normal; the 5 others

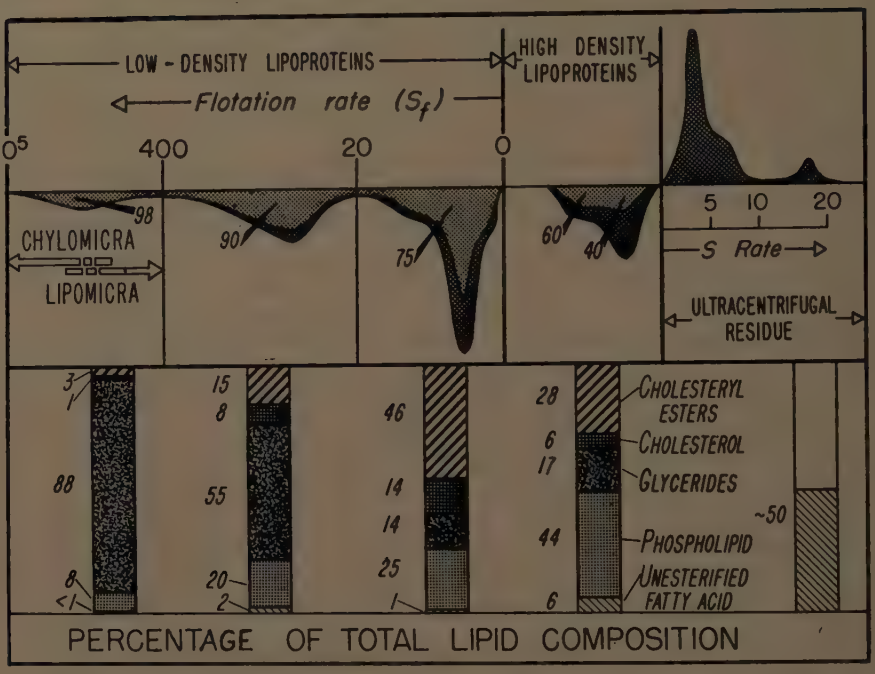

FIGURE 3. Lipid composition for the major broad bands of serum lipoproteins. The values of S_f 20 to 400, S_f 0 to 20, and major high-density lipoproteins represent the average for 9 fasting individuals. The S_f 400 to 10^5 are average values for 2 normal adults (see text).

were of such clinical abnormalities as to represent some of the most extreme types of low-density lipoprotein distributions known. Also included were the average compositions for 2 S_f 1500 to 10^5 chylomicra samples fractionated from 2 normal individuals approximately 2 hours after they had consumed 80 gm. of butterfat. Several lipoprotein lipid composition studies[10-11, 22, 23] have been reported on ultracentrifugally fractionated human sera. While somewhat different lipoprotein distributions were studied and different techniques employed for chemical analysis, no gross discrepancies in lipid chemistry have been described other than those for the clinical entity of biliary cirrhosis.[24]

If we consider separately each of the 9 cases studied for both the S_f 20 to 400- and S_f 0 to 20-class low-density lipoproteins, we find considerable variability in lipid composition within each broad lipoprotein group.

For the S_f 20 to 400 (FIGURE 4) the dominant lipid is consistently glyceride. All lipid components show variability, but particularly marked are the variations found in unesterified cholesterol and cholesteryl esters. For the S_f 0 to 20 class lipoproteins (FIGURE 5) there is considerable variability in cholesterol, cholesteryl ester, and glyceride content. A large part of the variability exhibited in lipid composition in both broad-band lipoprotein groups can be attributed to differences in lipoprotein distributions. However, from this

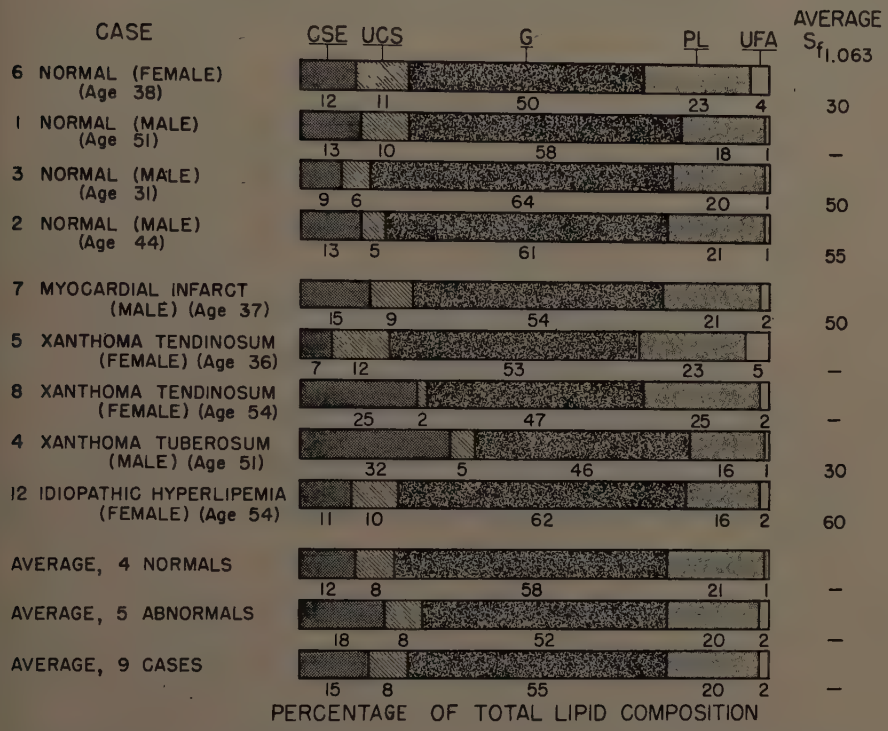

FIGURE 4. Lipid chemical composition of S_f 20 to 400 lipoproteins from fasting sera. Where available, the mean S_f rate of the total S_f 20 to 400 lipoprotein spectra is given. Abbreviations: CSE, cholesteryl esters; UCS, unesterified cholesterol; G, glycerides; PL, phospholipids; UFA, unesterified fatty acids.

broad-band data it is not possible to determine how much the observed differences in composition from person to person are the result of lipoprotein distribution differences and how much might reflect real and significant differences in composition (among individuals) of physically homogeneous regions isolated within such lipoprotein spectra. In a study of pooled plasma and serum this problem of lipoprotein distribution and composition within the low-density spectra has been considered in a study in which dextran sulfate was used as a precipitant to achieve convenient bulk isolation.[7]

The evaluation of lipoprotein homogeneity with respect to lipid composition. The problem of fractionating a relatively homogeneous lipoprotein band from regions of the serum lipoprotein spectra containing a continuous lipoprotein distribution has long been a difficult one. Recently lipoproteins have been successfully fractionated on nonequilibrium density gradients.[24, 25] However, the only theoretically stable density gradient in the preparative ultracentrifuge is the sedimentation-equilibrium salt gradient, which depends on the concentration and type of salt used under the specific centrifugal conditions. Equilibrium methodology[26] has been successfully applied to the analytic ultracentrifugal characterization of DNA in CsCl solutions. Sedimentation equilibrium has

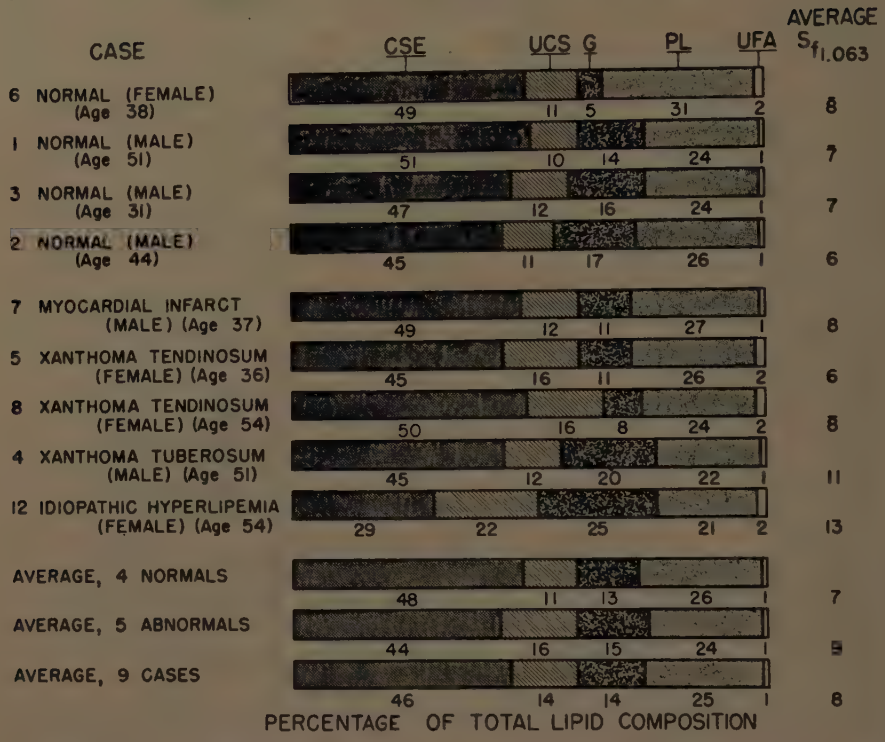

FIGURE 5. Lipid chemical composition of S_f 0 to 20 lipoproteins from fasting sera, including mean S_f values for each distribution. See caption to FIGURE 4 for abbreviations used.

been applied here for the preparative isolation of low-density lipoproteins of the S_f 6 to 8 class on approximate equilibrium gradients of NaBr set up in advance of the preparative run within a serum-NaBr system.

FIGURE 6 shows a run made on a salt solution whose NaCl and NaBr compositions were similar to that of the serum run. The broken line shows the density relationships in the preparative tube if layering occurred without mixing. The dotted line shows the gradient actually present as the tube was placed in the ultracentrifuge. The solid line is the gradient that existed at the conclusion of the 48-hour run, and it shows that in the region where slow equilibrium separation was involved very little small-molecule density change occurred. It should be noted that, after the first 12 hours of running, albumin and other high-density macromolecular contributors to effective density had passed into the lower half of the preparative tubes, leaving the approximate equilibrium gradient relatively undisturbed. Thereafter, in this central region of the tube a slow approach to equilibrium position occurs for the lipoproteins having hydrated densities equivalent to corresponding density regions on the equilibrium salt gradient. The establishment of complete equilibrium would require a run of several days. For the S_f 6 to 8 lipoproteins studied it was estimated that 80 per cent equilibrium had been achieved at the end of the 48-hour run.

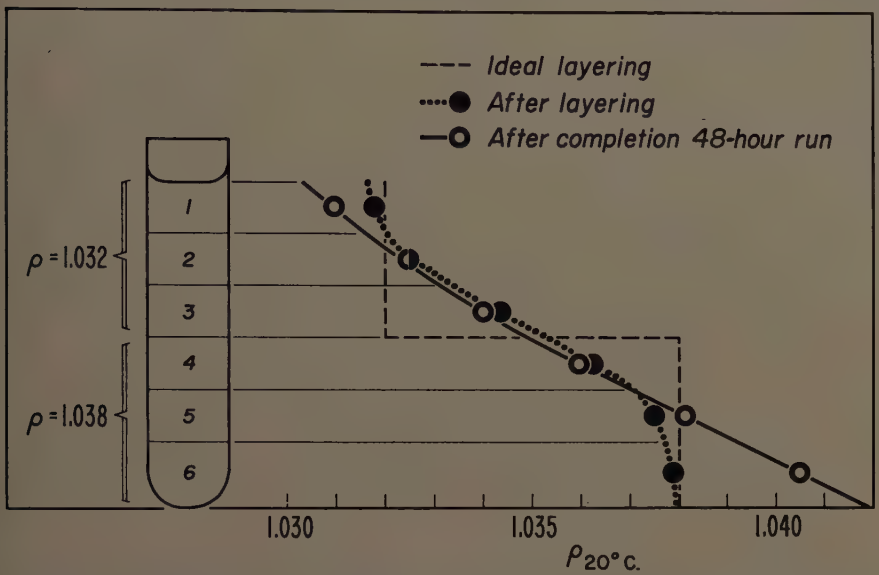

FIGURE 6. The density gradient utilized in the isolation of the S_f 6 to 8 lipoprotein band. The actual lipoprotein fraction studied in all 5 cases was the fourth milliliter, corresponding to a density region of 1.035 gm./ml.

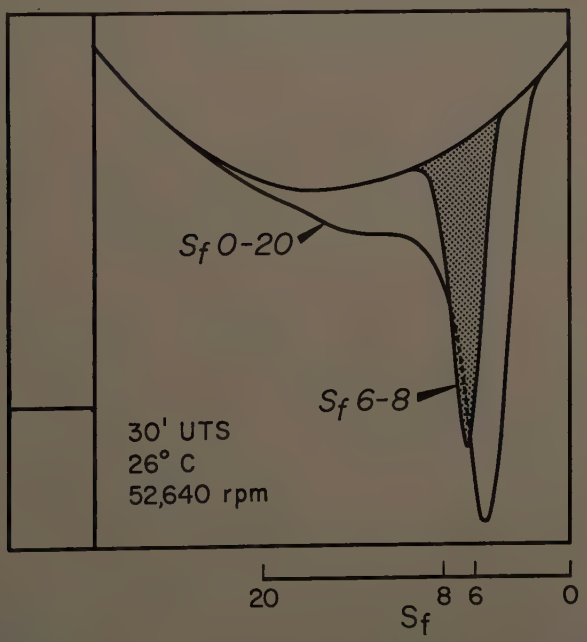

FIGURE 7. Comparison of the narrow S_f 6 to 8 lipoprotein band with the broad S_f 0 to 20 lipoprotein distribution from the normal 38-year-old male (third case). UTS is a symbol that indicates the time after the rotor reached "up to speed" that the Schlieren picture was taken.

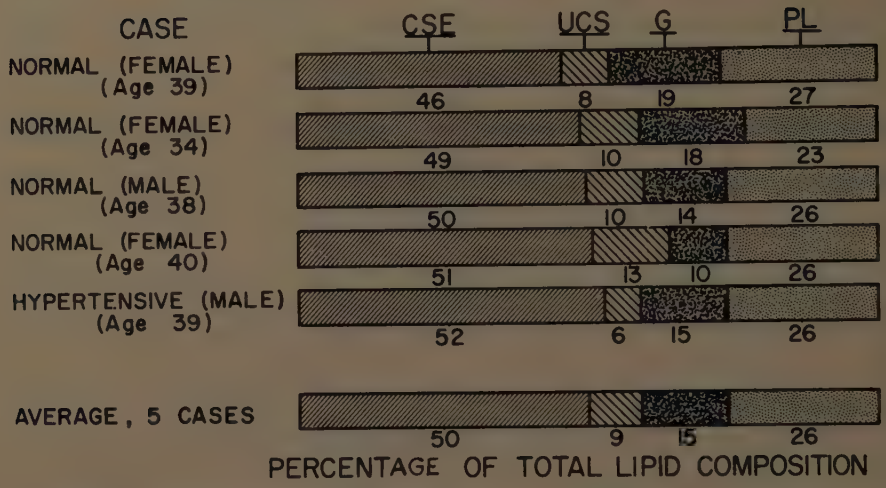

FIGURE 8. Comparison of the lipid compostion of the narrow band S_f 6 to 8 lipoproteins for 5 cases.

FIGURE 7 shows the results of lipid chemical analysis of narrow-band S_f 6 to 8 lipoprotein fractions obtained from 5 nonfasting adults (for a comparison of an S_f 6 to 8 narrow-lipoprotein band with the broad S_f 0 to 20 band fractionated from the same sera, see FIGURE 8). By comparison, there is considerably more uniformity of lipid composition than that found in the broad-band S_f 0 to 20 lipoprotein study, although each study evaluated different subjects, one group fasting and the other nonfasting. However, within the narrow S_f 6 to 8 lipoprotein band some variability appears to exist from person to person in both unesterified cholesterol and glyceride.

The amount of variability involved is considerable and represents several hundred molecules of such constituent lipid molecules as glycerides and cholesterol per lipoprotein molecule. Such variability might be the ultimate result of the dietary lipids previously eaten, metabolic factors, or both.

Low-Density Lipoprotein Structure

Apparent conversion of low-density lipoproteins and possible mechanisms of transformation. Any approach to the structure of the low-density lipoproteins, particularly those of higher molecular weight, must take into consideration certain dynamic relationships observed to occur among these large lipoprotein molecules. Lipoproteins of high S_f rate apparently convert into those of lower S_f values. This apparent conversion has been demonstrated by lipoprotein injection in both animals[27, 28] and humans.[29] Also, lipoprotein transformation[11, 30-32] occurs both *in vivo* and *in vitro* in the presence of a lipoprotein lipase system.[33, 34] Chemically, glycerides are hydrolyzed in this process.[35-37] Furthermore, all of these observed conversions within the low-density lipoprotein spectra are unidirectional from high S_f lipoprotein to lower S_f lipoproteins.

If the observed *in vivo* and *in vitro* conversions by heparin-activated lipo-

protein lipase bear any relationship to normal *in vivo* lipoprotein conversion, they suggest some restriction on the mechanism for such conversion. One such restriction might be that in lipoprotein conversion some substructural parts of one high S_f lipoprotein must be identical with substructural parts of lower S_f lipoproteins into which conversion is possible.

We do know that approximately 100 gm. of S_f 100 and higher lipoproteins (mostly above S_f 1000) are introduced daily into the blood stream of a normal adult as part of the thoracic lymph flow. These lipoproteins largely include S_f values from 400 to 75,000, corresponding to size ranges of from approximately 700 to 10,000 Å in diameter (assuming spheres). The metabolism of these lipoproteins necessarily involves a process either for their removal as a unit from the blood compartment or some rapid and efficient transformation mechanism in which their glyceride moiety is continuously hydrolyzed and removed from the blood compartment. Of course, a combination of these processes is also possible. Since there is evidence that some lipoprotein transformation with glyceride hydrolysis and fatty acid removal can and does occur within the blood compartment, this latter process may determine the sequence of molecular units through which, for example, an introduced S_f 2000 lipoprotein (of approximately 1600 Å in diameter) normally must proceed in order to unload, in a utilizable form (perhaps as a fatty acid albumin complex), its glyceride content. If a single S_f 2000 molecule was transformed successively (through many steps) into a single S_f 400, then into a single S_f 100 and, finally, into a single S_f 20 lipoprotein unit, one might estimate how much of the glyceride originally present in the S_f 2000 need be removed. Of course, a single S_f 2000 could give rise ultimately to many S_f 400, S_f 100, and S_f 20 lipoprotein units should the transformation process involve fissioning of the larger lipoprotein units. However, if the process were a surface hydrolysis without appreciable fissioning, the resulting S_f 400, S_f 100, and S_f 20 molecules would contain approximately 10, 1, and 0.1 per cent, respectively, of the glyceride present in the initial S_f 2000 molecule. In a sequence of unit lipoprotein conversion most of the glyceride hydrolysis might occur before yielding an S_f 400 molecule and nearly all before such conversion yielded an S_f 100 molecule. If we knew the mass relationships between lipoprotein groups in this long sequence we might estimate these relationships more closely, including estimations for extent of fissioning in this process. Without fissioning, however, much of the substructural protein and/or lipoprotein units would have to be discarded during the transformation process to yield normal product lipoproteins, with the appropriate protein content. This process of discarding substructural components might be selective. Thus, the protein moiety of a product lipoprotein, perhaps one order of magnitude smaller, might not necessarily closely resemble its parent protein moiety following transformation. Although unexplored, the physical process of fissioning may be an important aspect of low-density lipoprotein transformation.

Conversion of the lipid moiety. From the standpoint of the lipid moiety, the simplest consideration in the transformation of the high S_f low-density lipoproteins would involve only glyceride removal.

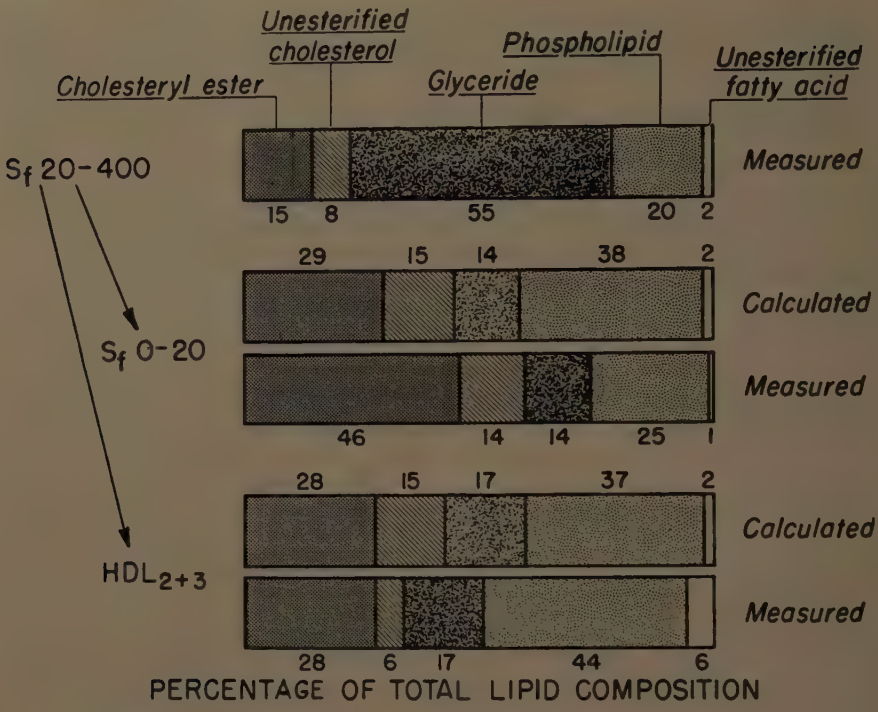

FIGURE 9. Results of glyceride removal (by calculation) from the S_f 20 to 400 lipoproteins High-density lipoprotein is abbreviated HDL.

If we mathematically consider conversion of the S_f 20 to 400 lipoprotein lipid moiety by glyceride removal to either the glyceride composition of the S_f 0 to 20 or the HDL_{2+3} lipoproteins, we find that the resultant composition resembles the high-density lipid composition more closely than the lipid composition of the S_f 0 to 20 lipoproteins (FIGURE 9). At first glance, this observation seems contradictory, in view of the data suggesting conversion of S_f 20 to 400 lipoproteins to S_f 0 to 20 lipoproteins. However, if the S_f 20 to 400 (and perhaps higher S_f as well) lipoproteins were to contain high-density lipoproteins as part of their structure, then this calculated relationship would be expected even though S_f 20 to 400 molecules might ultimately be converted into S_f 0 to 20 lipoproteins with substantially different protein moieties. It is possible that different substructural protein or lipoprotein units might participate in conversion within the S_f 0 to 20 class, yet not be involved in, for example, conversion within the S_f 20 to 100, S_f 100 to 400, or S_f 400 to 10^5 class.

Simplest model to explain conversion. FIGURE 10 illustrates a simple model for a high S_f lipoprotein that involves only a core of glyceride surrounded by high-density lipoproteins (estimated weight average for both HDL_2 and HDL_3 in the 45-year-old normal male). As an example, for a large spherical lipoprotein of diameter 1000 Å ($S_f \sim 760$) there would be in the neighborhood of 370

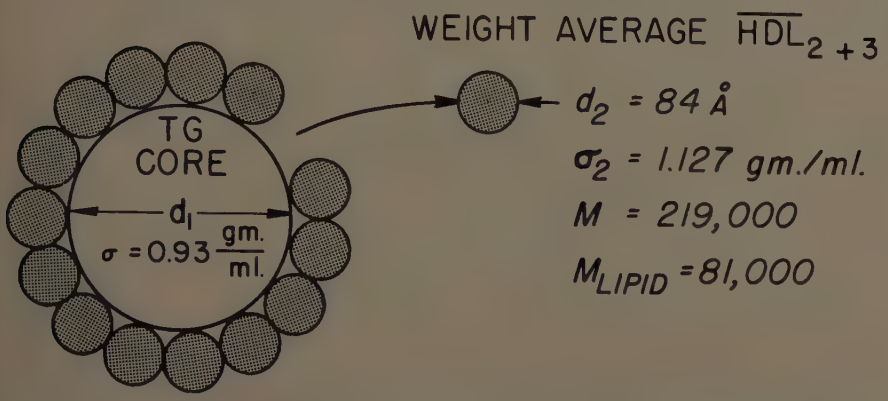

FIGURE 10. Simple model for the S_f 20 to 10^5-class lipoproteins. Abbreviations: HDL, high-density lipoprotein; TG, triglyceride.

closely packed spheres (each of diameter 84 Å) surrounding a glyceride core of 832 Å. This might be compared with another calculation that considers the principal high-density lipoprotein HDL_3 [38] as a prolate ellipsoid (40 × 150 Å). The approximate maximum number of such prolates that could fit around a sphere of 920 Å (longitudinally tangent to the sphere) would be approximately 360. In this model roughly the same number of high-density lipoproteins (spherical or prolate) might be associated to form this lipoprotein complex.

If we attempt to predict the composition of all low-density lipoproteins on this basis from the lipid chemical standpoint alone, this model definitely breaks down as we approach S_f 20 from the high-S_f side. FIGURE 11 illustrates a calculation of resultant lipid composition over the S_f 0 to 10^5 range derived by combining a glyceride core with an appropriate number of high-density lipoproteins sufficient to surround the glyceride core.

For lipoproteins above S_f 20 it is difficult to estimate the content of protein for a given S_f value. However, if we estimate the molecular weight for the protein moiety as a function of S_f value, we obtain the relation shown in FIGURE 12. It is clear that with increasing S_f, although the protein content is sharply decreasing, the molecular weight of the protein moiety is increasing enormously. Indeed, at about S_f 760 we should estimate the order of 0.9×10^7 molecular weight units of protein to be present. In our model we would need about 90 high-density lipoproteins, each having a protein molecular moiety of approximately 100,000, to provide the appropriate total protein molecular weight. In terms of this model there would be considerable exposure of the lipid moiety of the core. These estimates, of course, serve only to illustrate the reasonable quantitative aspect with regard to protein content of this simple model.

Methods to Trace Lipoprotein Conversion and Evidence for Substructure

During lipoprotein conversion it would be desirable to know what moiety or moieties might remain as an integral part or parts of the lipoprotein unit. Although possibly a temporarily associated unit, the protein moiety of the

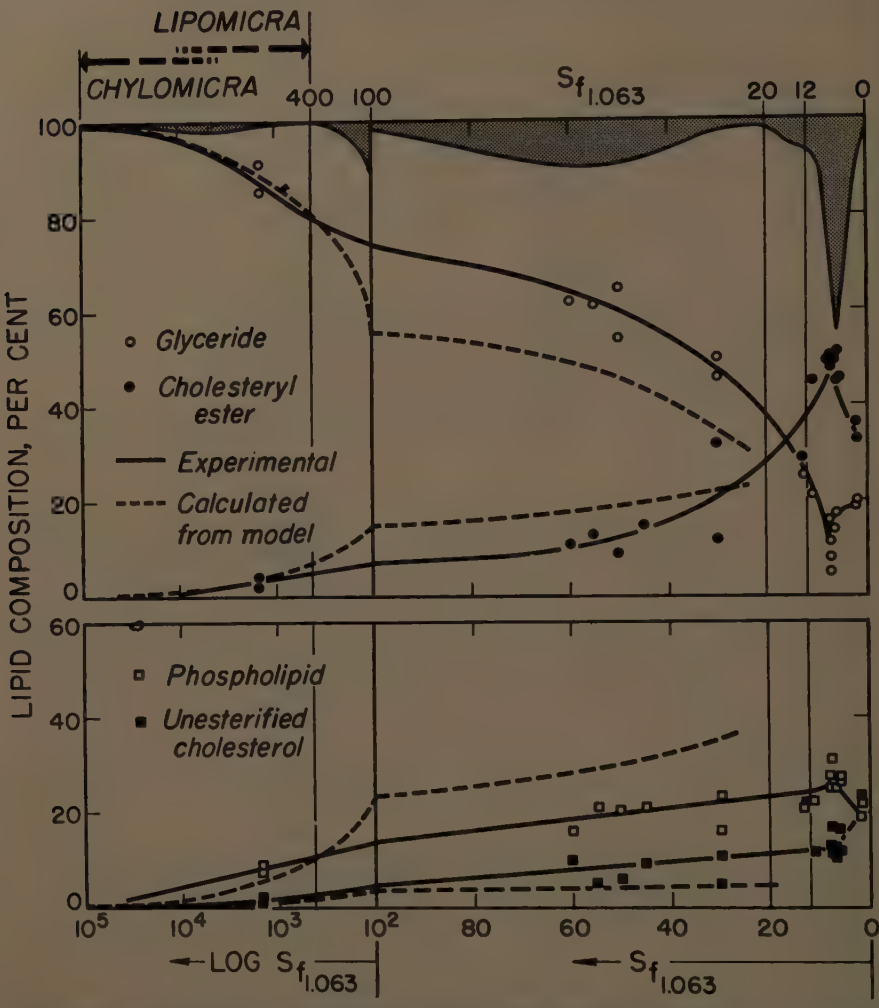

FIGURE 11. Comparison of the calculated lipoprotein lipid chemistry (using the simple model) with observed lipoprotein lipid composition over the range S_f 20 to 10^5. The discontinuity in the lipoprotein spectrum at S_f 100 results from the change (at this point) from a linear to a log S_f scale.

lipoprotein may be uniquely characterized by its N-terminal amino acid residues. FIGURE 13 shows the N-terminal characterization of all principal broad-band classes of the serum lipoprotein.

From these data[18-21] the protein moiety of each of the many classes of low-density lipoproteins appears to be a complex one. From the N-terminal data alone, it is not possible to say whether the protein moiety of such high S_f lipoproteins is composed of uncomplexed protein units or may be composed of many lipoprotein substructural units that are similar to or identical with other

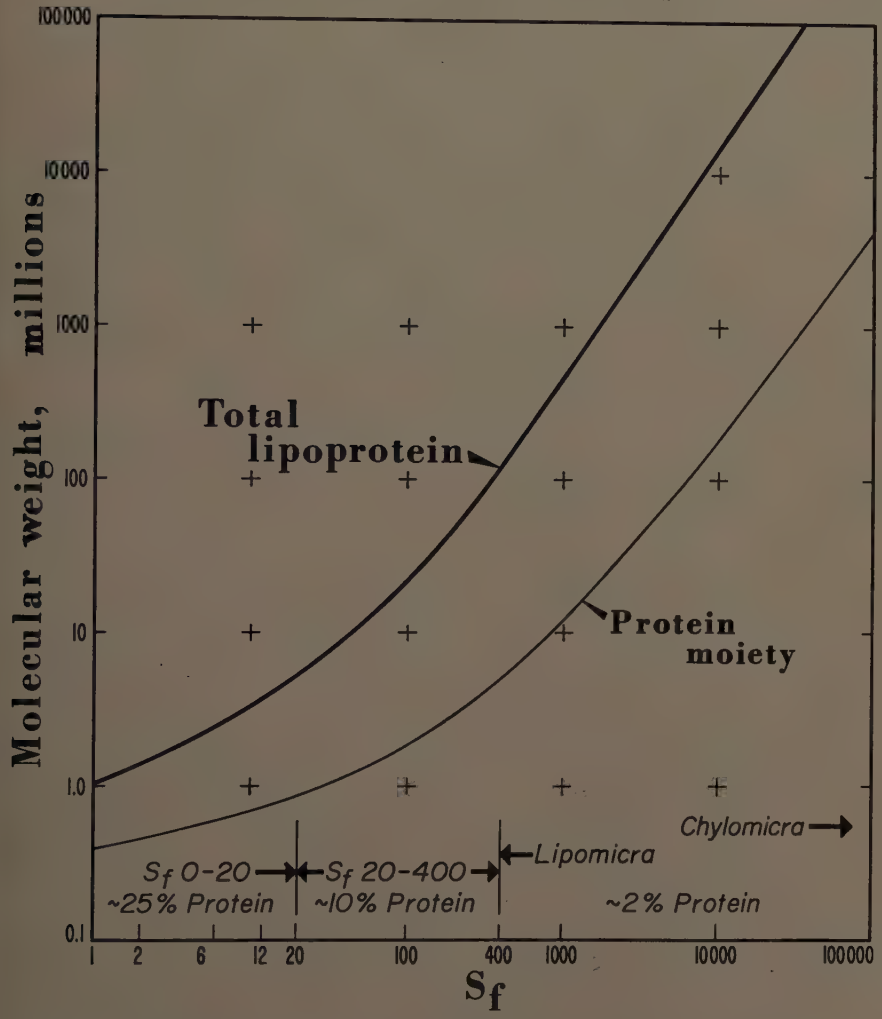

FIGURE 12. Lipoprotein molecular weight and molecular weight of the protein moiety estimated over the range S_f 1 to 10^5.

smaller freely circulating serum lipoprotein molecules. Furthermore, it appears that some protein or lipoprotein substructural units characterized by dominance of N-terminal serine and threonine are involved in the large low-density lipoprotein structures. This could be a high-density lipoprotein that exists freely in serum at relatively low abundance. More specific high-density lipoprotein subfractionation (possibly by equilibrium gradient techniques) followed by N-terminal characterization may clarify this point.

Degradation experiments on low-density lipoproteins have demonstrated that lipoprotein fragments (of higher density) are released during such proc-

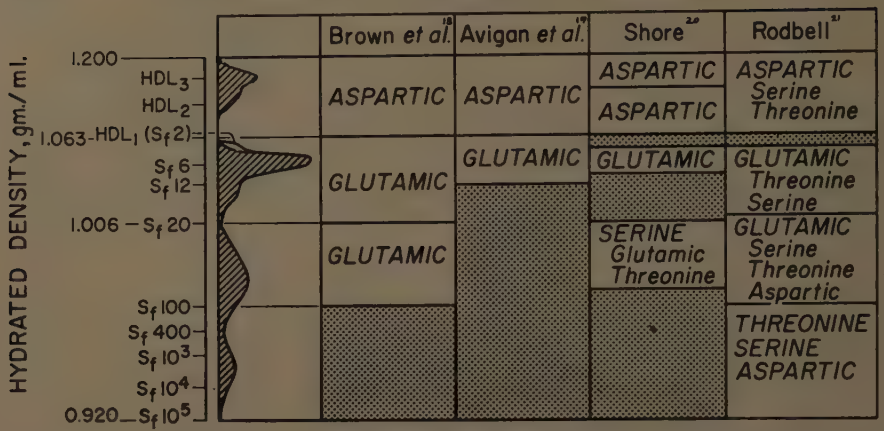

FIGURE 13. Summary of the N-terminal amino acid data,[18-21] allowing comparison of the lipoprotein groups studied. The dominant component or components are indicated in bold face, and the relatively minor components are shown in lower case. Trace components, although present in most cases, are omitted.

esses as prolonged dialysis,[39, 40] fatty acid degradation,[40] and ether degradation.[41-43] Hayashi *et al.*[42] found that the estimated molecular weight of the protein moiety of the high-density lipoprotein fragments was of the order of 400,000. Also in this same study small amounts of relatively lipid-free protein units of approximately 40,000 molecular weight units had been identified. However, in each of these experiments most fragments were actually lipoprotein units, although in many instances they were not similar either in lipid composition or molecular weight to normal lipoproteins of similar hydrated density.

In conclusion, a modified low-density lipoprotein model (FIGURE 14), taking into account some of these apparently conflicting factors, might have the following form.

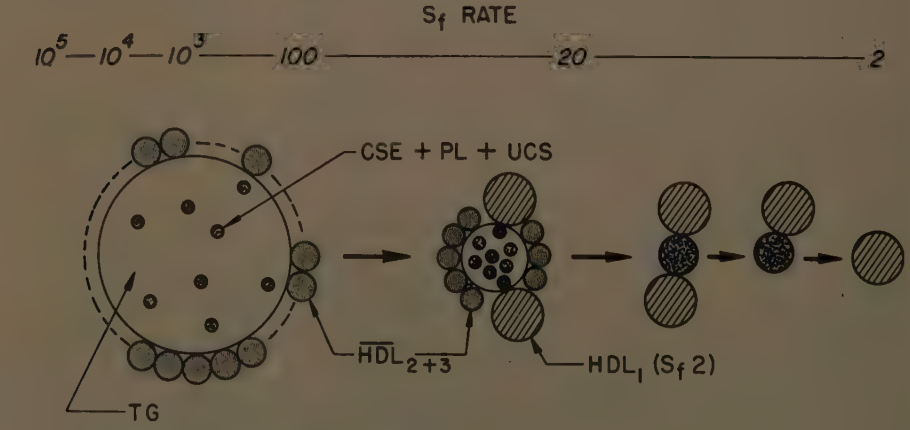

FIGURE 14. Modified S_f 2 to 10^5 lipoprotein model (see text for explanation).

First, the simple glyceride core would now contain small amounts of cholesterol, cholesteryl ester, and phospholipid. This would be more consistent with the actual composition of serum chylomicra, and perhaps the lymph chylomicra, which are known to contain lipid components other than glyceride, particularly phospholipid, in amounts that considerably exceed their protein content.[23] This composition could not be explained by a model composed of a simple glyceride core plus any combinations of smaller serum lipoproteins such as HDL_1 (S_f 2), HDL_2, or HDL_3. Reasoning on such a basis, it seems necessary to postulate a model consisting of a mixed lipid core (that is, glyceride plus small proportions of phospholipid and cholesterol) in order to explain the observed composition.

Association of any substructural small lipoproteins such as HDL_1 (S_f 2), HDL_2, and HDL_3 to form a lipoprotein complex in this revised model would have to yield the proper stoichiometry for each particular lipoprotein class as well as provide the proper net N-terminal amino acid picture for the total protein moiety.

Direct Visualization of Low-Density Serum Lipoproteins

Any hypothesis concerning the structual features of the many classes of low-density lipoproteins should agree with reliable physical data on these individual lipoprotein classes. In this connection, the electron microscope may be usefully employed to visualize directly all classes of the low-density lipoproteins.[44] The very large lipoproteins that are considerably greater than S_f 100 appear to be very nearly spherical in shape for the size range extending from approximately 800 to 10,000 Å (see FIGURE 15*a*). Preliminary work in this size range indicates rough agreement of electron microscope molecular weights with ultracentrifugal calculations, assuming spherical particles (which yield somewhat lower molecular weights). Structures between S_f 0 to 20 appear to be definitely flattened and represent interesting units that are more difficult to interpret directly. FIGURE 15 *b* shows an S_f 6 to 9 narrow-band lipoprotein preparation from a normal 42-year-old female. Calculations[45] based on shadow lengths and surface dimensions for these visualized structures yield molecular volumes of approximately 2 to 3 times that calculated from ultracentrifugal data (assuming spheres) or light scattering.[46] On close examination, these structures appear to be composed of two or possibly more asymmetrical subunits. If these subunits represent fundamental structures, they would be of the order of 3 million in molecular weight. Furthermore, to make a bundle composed of 2 or more units, the primary molecular unit would probably have to be asymmetrical to the extent of at least 2:1 in axial ratio (if prolate ellipsoids or long cylinders are assumed). Another possible explanation of these large visualized structures seen in FIGURE 15 *b* consistent with other molecular-weight data could be an unusual single unit resembling a biconcave disk or torus.

However, using spray techniques[47] recent electron microscope studies[48] of similar S_f 6 to 8 lipoprotein preparations (in which were incorporated concentrations of polystyrene latex with accurately known physical properties)

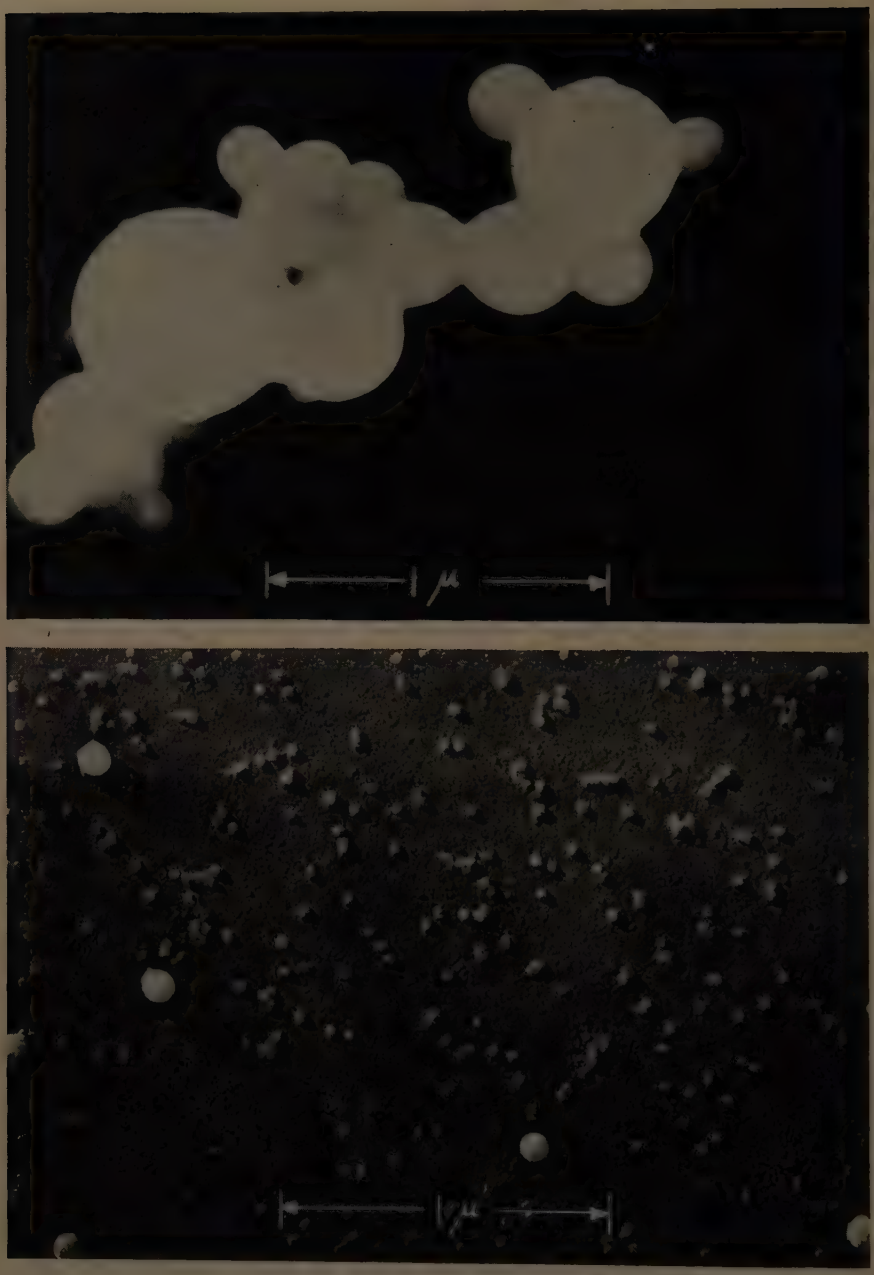

FIGURE 15. Electron micrographs of low-density human serum lipoproteins. These macromolecules were isolated ultracentrifugally, fixed in 1 per cent OsO_4, shadowed with a Pt-Pd-Au alloy, and viewed in an RCA EMU 2-E electron microscope.[43] Above, S_f 100 to 10^5 lipoproteins. × 69,000. Below, S_f 6 to 9 lipoproteins, including several 880-Å polystyrene latex marker molecules. × 65,000.

have given by direct particle count molecular weights in the range of from 6 to 9 million units. Such data, in combination with the light-scattering and ultracentrifugal molecular weight determinations, strongly suggest that these visualized structures are composed of two or three asymmetrical subunits, the longest dimension of which is in the neighborhood of 350 Å. This preliminary conclusion would not be in conflict with the light-scattering data relating both to the molecular weight and shape of these S_f 6 to 8 lipoproteins. Such data[46] obtained from several samples of S_f 6 to 8 lipoproteins yielded molecular weights of from 2.8×10^6 to 3.1×10^6. Furthermore, calculations from dissymmetry data yielded prolate ellipsoids of approximate dimensions of 160×360 Å.

These asymmetrical shapes considered for S_f 6 to 9 lipoproteins are different from the spherical shape given previously[49] for chemically isolated β_1 lipoprotein (S_f 2 to 10).

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CHYLOMICRONS AND LIPID TRANSPORT

J. H. Bragdon
National Heart Institute, Public Health Service, Bethesda, Md.

Most ingested fat is absorbed from the intestine in the form of chylomicrons in the chyle. By cannulating the thoracic ducts of animals and feeding them labeled long-chain fatty acids it is easy to recover labeled chylomicrons. These chylomicrons can then be injected intravenously into other animals, where their fate can be determined. In this way the processes of distribution can be separated from those of absorption. Another component of the blood lipids, small in concentration but physiologically very active, are the unesterified fatty acids bound to albumin (UFA). Labeled fatty acids bind readily to albumin *in vitro* and can be injected intravenously in animals and man in amounts sufficient to be followed. For several years our laboratory has been utilizing these techniques in the study of lipid transport.

Chylomicron Composition

The chylomicron consists predominantly of triglycerides, plus small amounts of cholesterol, phospholipids, and protein. Its cholesterol content can be increased by cholesterol feeding, but its phospholipid content cannot be increased by feeding phospholipids. This is illustrated in TABLE 1, which shows the percentage composition of chylomicrons after feeding various lipids to a rat. TABLE 1 also shows the total chylomicron lipid in 5 ml. of chyle during each feeding period. The fact that this was the same after feeding lecithin as after feeding olive oil indicates that the fed lecithin was hydrolyzed largely in the intestine, that the fatty acids were incorporated into chylomicron triglyceride, and that the phosphorus-containing moiety was absorbed primarily via the portal vein.

There is no uniformity of opinion concerning the protein content of chyle chylomicrons. Albrink *et al.*[1] state that if protein were present at all it would be in amounts too small to measure. Robinson[2] stated that the small amount present on original separation could be removed completely by repeated centrifugal washings through saline, implying that the protein was not an integral part of the chylomicrons, but represented an adsorption phenomenon. TABLE 2 shows that the protein content, about 0.5 per cent by dry weight, remains constant through successive washings through saline. It is believed that Robinson failed to detect the protein because of unsuitable methodology.

Chylomicrons are far from homogeneous. The chemical composition of any preparation depends to some extent on the centrifugal conditions used for its separation. The preparation referred to in TABLE 2 was separated from whole chyle by layering the latter under physiological saline and centrifuging it for 30 min. at 100,000 g. This is sufficient force to pack out all the light-scattering particles in a butterlike mass at the top of the tube. If, on the other hand, the chyle is layered under saline and centrifuged more gently, as suggested by Lindgren *et al.*,[3] that is for only 10 min. at 9000 g, only a small part of the light-

TABLE 1

EFFECT OF DIET ON LIPID COMPOSITION OF CHYLE CHYLOMICRONS

Diet	Total lipid* (mg.)	Cholesterol (percentages)	Phospholipid (percentages)
Mash + olive oil	198	0.66	6.9
Mash + soy lecithin	201	0.43	6.8
Mash + olive oil + cholesterol	219	1.9	7.2

* In chylomicrons from 5 ml. of chyle.

TABLE 2

THE EFFECT OF REPEATED WASHINGS ON THE PROTEIN CONTENT OF RAT CHYLE CHYLOMICRONS

No. of washes	Protein (percentages)
2	0.45
3	0.46
4	0.45
5	0.45

scattering particles solidify at the top of the tube. These are the largest chylomicron particles with the very lowest densities. They show a lower content of phospholipid and of protein, but the protein content of several such preparations has never been less than 0.2 per cent. Assuming the inhomogeneity of this fraction, it is of course possible that some chylomicrons are actually protein-free.

This amount of protein is apparently inadequate to cover the surface of a sphere 0.5 μ in diameter. If we accept the data compiled by Bull,[4] which show that proteins spread in a monolayer occupy an area of 0.78 m.^{2}/mg. protein, then chylomicrons 0.5 μ in diameter must contain at least 1.5 per cent protein if the entire surface is to be covered. It is quite likely that the stability of these lipid emulsions is produced by the phospholipids that they contain rather than by the protein. Robinson[2] has shown that emulsions of rat chylomicrons are completely disrupted by small amounts of *Clostridium welchii* lecithinase.

Origin of Chylomicron Protein

We have recently become interested in the nature and source of the chylomicron protein. If this protein were newly synthesized in the intestinal wall, the feeding of labeled amino acids during active fat-absorption should produce a very high specific activity in the chylomicron protein immediately after feeding, and this peak should fall off rapidly as the fed amino acids became absorbed and diluted in the body pool. Cannulated rats that were eating pellets saturated with olive oil were tube-fed a mixture of C^{14}-labeled amino acids. FIGURE 1 shows the results of one such experiment. The specific activities of the chylomicron protein, of the chyle lipoprotein protein, and of the

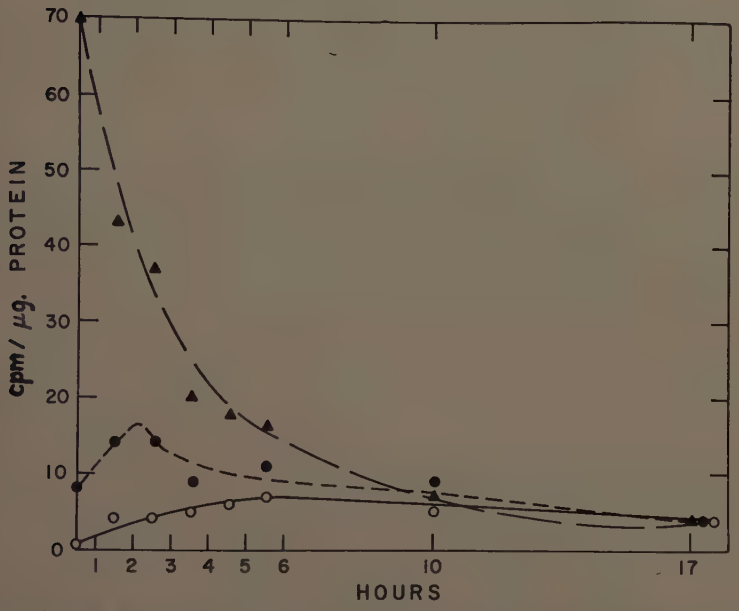

FIGURE 1. The specific activities of chylomicron protein (▲), lipoprotein protein (●), and residual proteins (○) of the chyle after the feeding of C^{14}-labeled amino acids in a rat. In each case the points have been plotted midway through the collection period.

residual chyle proteins are plotted against time. The label appears in very high concentration in the chylomicron protein during the first hour after feeding and drops off rapidly thereafter. The lipoproteins and the residual proteins show much lower specific activities, and the peaks are attained only after several hours. These labeled lipoproteins and residual proteins probably entered the chyle from liver lymph and represent liver synthesis, for it has been demonstrated recently in our laboratory that the isolated perfused liver incorporates labeled amino acids into both alpha and beta lipoproteins, as well as into the residual proteins of the perfusing plasma. These specific activity data of chylomicron protein strongly suggest, although they do not prove, that at least part of the chylomicron protein is synthesized in the intestinal wall. Rodbell and Fredrickson[5] have recently been able to dissolve delipidated chylomicron protein in urea and to subject the solution to paper electrophoresis. These investigators have shown in chylomicron protein from the dog and from man that each contains 3 distinct proteins, one of which appears to be the same protein that exists in alpha lipoprotein. This has been shown by the identical patterns that appear after paper chromatography of the partially hydrolyzed proteins. Rodbell and Fredrickson have shown further that this particular chylomicron protein develops the highest specific activity in the dog after the feeding of labeled amino acids. It thus appears likely that part of the chylomicron protein is the same protein as that in alpha lipoprotein, and that this protein can be synthesized both by intestinal wall and by liver. It is of considerable interest in this regard that Korn[6] has shown

that alpha lipoprotein was the only serum protein that would "activate" a synthetic coconut oil emulsion, that is, would convert it into a suitable substrate for the clearing-factor enzyme lipoprotein lipase.

Fate of Chylomicrons

To investigate the fate of chylomicrons once they have entered the blood stream, Havel and Fredrickson[7] injected dogs with chylomicrons labeled with palmitic acid-1-C^{14}. Ninety-seven per cent of the label was in the triglycerides; the remainder in the phospholipids. These investigators showed that the chylomicrons were removed rapidly from the blood stream, and that the label promptly reappeared as unesterified fatty acid bound to albumin (UFA), although there was no actual increase in UFA concentration. The amount of label that appeared in the other lipoproteins of the sera of the recipients was negligible. These investigators concluded that the chylomicrons probably were being removed intact from the blood stream by some tissue, and that hydrolysis of the triglycerides took place there with the subsequent release of the fatty acids back into the blood stream.

Several factors affecting the rate of clearance of chylomicrons have been studied. French and Morris[8] have shown that the removal rate is exponential, but that increases in the dose increase the plasma half-life. The removal rate has been shown to be independent of the nutritional state of the animal.[9] Rats on diets very high in carbohydrate cleared chylomicrons from the blood just as rapidly as did fasting animals. The clearance rate, at least of large doses, can be increased by heparin and reduced by protamine,[10] suggesting that the clearing factor enzyme, lipoprotein lipase, plays a role in the removal mechanism.

Although the nutritional state of the animal has no significant effect on the removal rate, it has a profound effect on the ultimate oxidation of the fatty acids of the chylomicrons. FIGURE 2 shows the cumulative $C^{14}O_2$ excretion in 2 fasting and 2 carbohydrate-fed rats during 90 min. following the injection of chylomicrons containing C^{14} in palmitic acid. About 45 per cent of the dose was excreted by the fasting animals, as compared with about 5 per cent in the fed rats. This approaches very closely the oxidation rate following the injection of labeled palmitic acid as UFA.[11]

The nutritional state of the animal also apparently has some effect in determining the anatomic sites at which chylomicrons are removed from the blood.[12] Rats were injected with palmitic acid-1-C^{14}-labeled chylomicrons. They were sacrificed 10 min. later, and the amount of label in the several tissues was determined. In the fasting animal most of the chylomicrons were cleared by the liver with significant amounts in the muscle; in fact, the heart muscle contained just as many counts on a weight basis as did the liver. This is of interest in view of the fact that rat heart is an excellent source of lipoprotein lipase.[13] In the carbohydrate-fed animals, on the other hand, although the liver contained many counts, one third of the cleared activity was found in the fat depots. Because we know from the work of Havel and Fredrickson[7] that during the clearing process the fatty acids of the chylomicrons rapidly appear

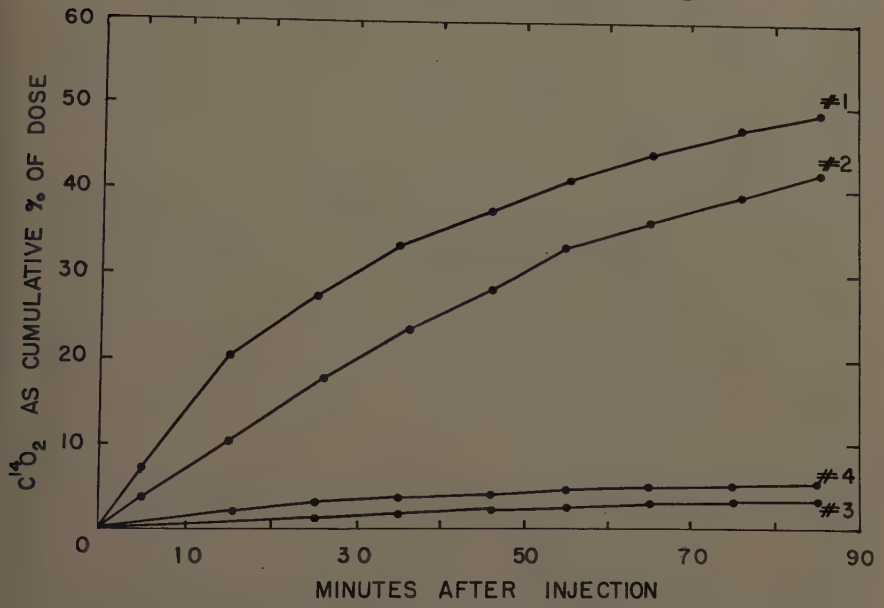

FIGURE 2. $C^{14}O_2$ excretion plotted as cumulative percentage of the dose administered as labeled chylomicrons. Rats Nos. 1 and 2 were fasting; rats Nos. 3 and 4 had been fed carbohydrate.

as UFA, it was necessary to inject other rats with labeled UFA in order to determine its tissue distribution. If intravascular hydrolysis of triglyceride to UFA, as has been suggested by others,[14] were the principal pathway for the clearance of chylomicrons, one would expect the distribution of labeled fatty acid to be the same, whether injected as chylomicron or as UFA. Such was not the case. After chylomicron injection into carbohydrate-fed animals, about one third of the cleared counts were found in the body fat, whereas after UFA injection less than 4 per cent of the cleared activity appeared there, proving that chylomicrons were being removed intact by adipose tissue without prior hydrolysis.

UFA Metabolism

Fredrickson and Gordon[15] have recently completed studies on UFA turnover and oxidation in humans. They have injected tracer amounts of C^{14}-labeled fatty acids bound to albumin intravenously and have followed the disappearance of the label from the blood and its appearance as $C^{14}O_2$. The initial phase of the plasma-disappearance curve is exponential and very steep. Its slope depends to some extent on the actual UFA concentration in the individual. The plasma UFA is in equilibrium with a fatty acid tissue pool. The latter is not sufficiently large to include the total adipose stores of the body. The average fasting individual turns over about 0.75 mEq. of fatty acids per minute, which is the amount in 1 l. of plasma. This is a sufficient amount of fat to satisfy the entire caloric needs, but these investigators have subse-

quently determined that only a portion of the plasma UFA being turned over is directly oxidized. A large part of the labeled UFA recycles one or more times. This still leaves the UFA with a very important role in fat transport and metabolism.

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DISCUSSION OF PART I

ROBERT G. LANGDON (*Johns Hopkins University School of Medicine, Baltimore, Md.*): Currently some of the major problems of biochemical interest are related to questions of how the integration and control of metabolism, particularly hormonal control, are achieved at the molecular level. In order to approach these problems intelligently, it seems obvious that we must have detailed chemical knowledge of the processes that are being controlled. The excellent papers of Rossiter and Staple have very clearly defined the present state of our knowledge in the areas of phospholipid and triglyceride synthesis, as well as the biosynthesis and intermediary metabolism of the steroids, and these investigators have laid a foundation upon which rational approaches to the complex problems associated with the control and integration of the metabolism of these lipids can be based.

Biochemists frequently have been accused, with some justification, of regarding the cell as a bag full of enzymes, a grab bag from which enzymes and enzyme systems are plucked rather capriciously and completely out of their context of cellular organization. However, what these critics have often not considered is that initial oversimplification of a complex process frequently provides the experimental key to the eventual understanding of the over-all process. As evidenced by the papers presented by Rossiter and Strickland and by Staple, considerable progress has been made in this initial step.

However, I believe that we must admit that even fairly detailed understanding of the intermediate events occurring in a metabolic sequence, although necessary, may be insufficient to delineate the mode of operation of any biological controls that may be exerted upon the over-all process because there are parameters, such as intracellular localization of the process and its integration with ancillary enzymes, that may require consideration. To provide more concrete illustration of this point, I shall consider very briefly some aspects of fatty acid oxidation and synthesis. Fatty acid oxidation and synthesis are diagrammatically depicted in FIGURE 1. As shown there, the oxidation of fatty acyl CoA esters proceeds in the mitochrondria by a now well-known sequence of reactions. I particularly call attention to the first step in this sequence, the oxidation of a saturated fatty acid by a flavoprotein dehydrogenase. In the mitochondria this reaction appears to be irreversible under physiological circumstances. This may be partially or completely responsible for the observed inability of mitochondria to synthesize long-chain fatty acids.

Fatty acid synthesis, on the other hand, does occur extramitochrondrially in the soluble cytoplasm by a sequence of reactions resembling those of fatty acid oxidation. However, the final reductive step in which α,β-unsaturated fatty acids are reduced to saturated fatty acids by reduced triphosphopyridine nucleotide (TPNH) is a thermodynamically irreversible reaction, and the process of fatty acid synthesis appears to be irreversible at this intracellular site. From this we may conclude that fatty acid oxidation and synthesis occur at different intracellular sites and that each process is irreversible at the site at which it occurs. These concepts have been very helpful in explaining the

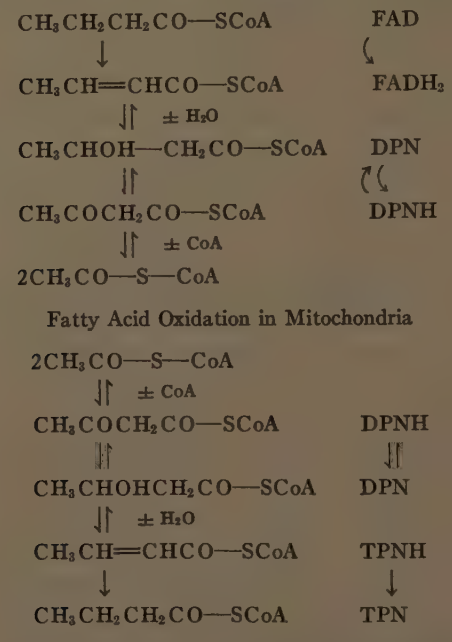

FIGURE 1. Fatty acid oxygen and synthesis. Symbols: FAD, flavine adenine dinucleotide; FADH, FAD-reduced; DPN, diposphopyridine nucleotide; DPNH, DPN-reduced; TPN, triphosphopyridine nucleotide; TPNH, TPN-reduced.

characteristic dissociation of fatty acid oxidation and synthesis observed in diabetes mellitus. It is apparent that they not only can but must be dissociated because they are in reality separate and unrelated entities.

It is becoming increasingly apparent that the synthesis and degradation of most major tissue constituents are dissociated in that they are carried out either at different intracellular sites, by different enzymatic reactions, or both. It seems that synthesis and degradation represent the traffic in parallel one-way streets of opposite direction and that each is irreversible because of the presence in each sequence of one or more irreversible steps. Examples of this situation are the synthesis and catabolism of cholesterol, glycogen, triglycerides, phospholipids, porphyrins, and probably proteins. As a result, the net quantity of a tissue or serum constituent does not reflect a mass law relationship, but is the resultant of (at least) two independent unidirectional processes proceeding simultaneously in opposite directions. It is obvious that the possibilities for biological control are much more favorable under these conditions than they would be if the balance between catabolism and synthesis were determined directly by mass law considerations in a two-way channel.

CARLETON R. TREADWELL (*George Washington University School of Medicine, Washington, D. C.*): I direct my remarks chiefly to the material presented by

Swell. One implication of the data presented is that the esterification of cholesterol in the intestinal wall is an essential step in the transfer of cholesterol from the lumen to the lymph. The absorption of epicholesterol entirely in the free form has been cited as showing that esterification is not essential for sterol absorption. While this absorption of epicholesterol without esterification can perhaps be explained by the incorporation of epicholesterol in the chylomicron in place of free cholesterol, the absorption of this compound certainly merits further study.

If I understand correctly the mechanism recently proposed by Glover and Green,[1] it does not account in its present form for the appearance of labeled cholesterol in the lymph for periods of several days after a single administration; also, it does not account for the obligatory requirement for bile salts, demonstrated by Siperstein and his co-workers,[2] or for the loss of the capacity to absorb cholesterol in the animal with a fistula of the pancreatic duct. Glover and Green[1] state that the esterification of cholesterol accelerates the transfer of cholesterol from the mucosa into the lymph; why or how this should be the case is not clear. Similarly, in the mechanism proposed by Swell there is still considerable uncertainty regarding the nature of the esterification step. While there are data from our laboratory[3] and that of Chaikoff[4] suggesting that the cholesterol esterase system is involved in the synthesis of the cholesterol esters in the mucosa, it is still possible that this synthesis involves some form of activated intermediate.

One of the most attractive aspects of the mechanism proposed by Swell is that there appears to be some unity in the absorption of fats and sterols. The interrelationships between triglycerides, phospholipids, and cholesterol during absorption is another aspect that requires further study, but the mechanism presented places emphasis on the importance of the chylomicron and its formation in absorption. The data presented by Bragdon show that the chylomicron contains a unique protein and that, from the standpoint of absorption, chylomicron formation must be an extremely complex process.

If we regard the absorptive process as involving the synthesis of triglycerides, phospholipids, cholesterol esters, and protein in the mucosa and their regulated incorporation into the chylomicron, it is clear that a broad spectrum of metabolic activity in the mucosa is involved in absorption, much of which remains to be investigated. If all of these synthetic processes are involved in absorption, it may be profitable to reconsider the possibility that these processes are under some degree of endocrine control, as suggested nearly twenty-five years ago by Verzar.[5]

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Part II. Pituitary and Adrenal Hormones: Lipids and Arteriosclerosis

THE ROLE OF THE PITUITARY AND THYROID IN DCA-INDUCED CARDIOVASCULAR DISEASE IN THE RAT

E. D. Salgado* and M. I. Mulroy
Pfizer Therapeutic Institute, Maywood, N. J.

The administration of desoxycorticosterone acetate (DCA) to rats induces a syndrome whose functional (arterial hypertension) and morphologic (cardiovascular and vascular lesions) symptoms are reminiscent of the human hypertensive disease.[1]

In this paper, data showing the profound influence exerted by the pituitary and thyroid on DCA-induced cardiovascular disease are discussed.

General Comments on Techniques

Selye *et al.*, in 1943,[1] showed that daily administration of DCA was followed within 3 weeks by polyuria, hypertension, nephrosclerosis, and periarteritis nodosa. Several years later, Prado[2] and others[3-5] found that if the treatment were continued for several weeks it then could be stopped, producing the so-called "meta-DCA" hypertension, and the high blood pressure would not return to normal levels.

These techniques can be reproduced easily, but in our experience, they may be modified so as always to obtain high blood pressure and yet affect the incidence and severity of the other symptoms. Examples of these modifications are given below.

To produce the disease in a short period of time, we use young rats weighing 35 to 50 gm., unilaterally nephrectomized, implanted subcutaneously with 1 to 5 pellets of DCA, and offer them ad libitum 1 per cent NaCl as their sole drinking fluid. The animals are sacrificed during the third or fourth week of treatment. At this time (TABLES 1, 2, and 3, and FIGURE 1) they show hypertension and severe lesions in the heart and kidney, some lesions in the brain, and a few or no lesions in the mesenteric arteries. The general condition of the animals depends on the amount of DCA given (1 to 5 pellets), perhaps because the higher the dose of DCA the more severe the hypopotassemia.

A completely different picture is obtained with our technique of producing "meta-DCA" hypertension, as illustrated below.

A group of 100 male Sprague-Dawley rats, 30 days old, were implanted subcutaneously with a 25-mg. pellet of pure DCA and offered ad libitum 0.87 per cent NaCl as drinking fluid. Fifteen weeks later 70 animals were alive and were divided into 3 groups. Group 1 was sacrificed, Group 2 was offered only tap water for drinking, and Group 3 was continued on saline as drinking fluid. The remainder of the DCA pellet was removed in Groups 2 and 3. Blood pressures were taken at intervals until the experiment was terminated at week

* Present address: Department of Pathology, Seton Hall College of Medicine and Dentistry, Jersey City, N. J.

TABLE 1

INFLUENCE OF THYROPARATHYROIDECTOMY ON TISSUE LESIONS OF RATS* WITH HIGH OR LOW DOSES OF DCA

Lesions in:	Controls		DCA (1 pellet)		DCA (5 pellets)		DCA (1 pellet) + Thyroparathyroidectomy†		DCA (5 pellets) + Thyroparathyroidectomy†	
	Incidence	Severity	Incidence	Severity	Incidence	Severity	Incidence	Severity	Incidence	Severity
	percentages									
Brain	0	0	33	83	50	87	0	0	0	0
Heart	0	0	67	83	77	100	0	0	22	100
Kidney	0	0	83	73	80	79	20	33	44	75
Mesenteric arteries	0	0	0	0	10	100	30	100	44	100

* All the animals were unilaterally nephrectomized on day 0, drank 1 per cent NaCl, and were sacrificed on day 26.

† On day −3.

TABLE 2

INFLUENCE OF DRINKING FLUID ON SURVIVAL AND LESIONS OF META-DCA HYPERTENSIVE RATS*

Lesions in:	Group 1 Sacrificed at 15 weeks, 20†		Group 2 H_2O Sacrificed at 40 weeks, 25 (20)†		Group 3 0.87 per cent NaCl Sacrificed at 40 weeks, 25 (12)†	
	Incidence	Severity	Incidence	Severity	Incidence	Severity
	percentages					
Heart	15	33	10	33	25	33
Kidney	35	39	20	29	50	87
Mesenteric arteries	95	68	75	63	42	53

* For details see text.

† Initial and final (in parentheses) number of rats.

40. As may be seen in FIGURE 2 and TABLE 2, treatment of rats with small doses of DCA (the average daily absorption from our pellets was 130 μg. of DCA) results in self-sustained hypertension. Substitution of saline for water after hypertension became established did not influence blood pressure (similar results were obtained by Green *et al.*[4] and recently by Gross[6] and by Sturtevant (personal communication). Survival was improved, and there was also a significant improvement in the lesions in the kidneys and mesenteric arteries. In the mesenteric arteries the animals on saline (Group 3) showed simultaneously acute and chronic lesions (FIGURE 3), whereas the animals on water (Group 2) showed only chronic lesions in the healing stage. Similar but more severe lesions can be obtained by giving 3 DCA pellets instead of 1 or 1 per cent instead of 0.87 per cent NaCl, or both (see DCA controls in TABLE 4 and FIGURE 4). We believe that our technique is unique in that it results in a high percentage of periarteritis nodosa.

TABLE 3

INFLUENCE OF THYROPARATHYROIDECTOMY AND THYROXINE ADMINISTRATION ON TISSUE LESIONS OF DCA-TREATED* RATS†

Lesions in:	Control		DCA		DCA + Thyroparathyroidectomy‡		DCA + Thyroparathyroidectomy‡ Thyroxine§	
	Incidence	Severity	Incidence	Severity	Incidence	Severity	Incidence	Severity
	percentages							
Brain	0	0	25	100	0	0	14	16
Heart	0	0	75	50	0	0	43	50
Kidney	0	0	87	59	0	0	43	88
Mesenteric arteries	0	0	12	16	44	91	0	0
Hypophysis, mg. per cent	3.1		3.3		4.5		3.4	

* Three 25-mg. pellets.

† All the animals were unilaterally nephrectomized on day 0, drank 1 per cent NaCl, and were sacrificed on day 29.

‡ On day −3.

§ 3.75 μg./rat/day/subcutaneously.

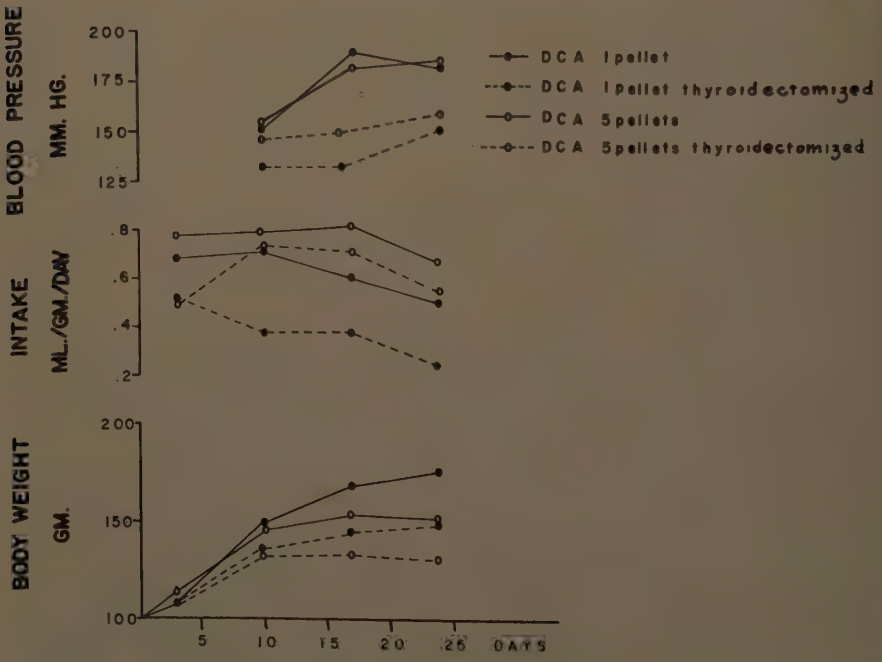

FIGURE 1. Changes in blood pressure, fluid intake, and body weight of rats receiving DCA. See TABLE 1 for details.

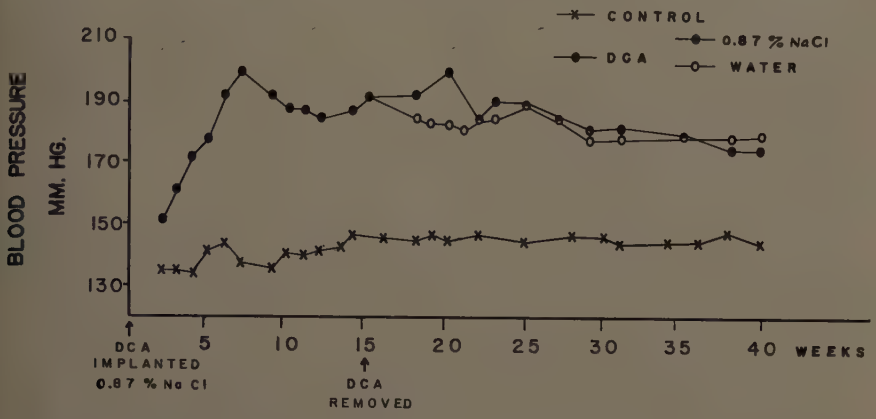

FIGURE 2. Changes in blood pressure of rats receiving small doses of DCA. For details see text and TABLE 2.

Influence of the Pituitary

Hypophysectomy causes a fall of blood pressure in renal hypertensive dogs.[7, 8] This fall is due to the absence of the anterior lobe, since the extirpation of the posterior lobe is without effect.[9] It is nevertheless possible to produce renal hypertension in the absence of the gland if the renal arteries are constricted 1 month after removal of the pituitary.[8, 10, 11] It was concluded[11] that renal hypertension is independent of the pituitary.

The removal of the hypophysis prior to DCA implantation does not prevent the rise in fluid intake, but prevents the hypertension, the cardiorenal lesions, and the lesions in the mesenteric vessels.[12, 13] The same is true for hormonal hypertension produced with somatotrophin (STH) or adrenocorticotrophin (ACTH).[14] Treatment with lyophilized anterior pituitary (LAP),[15] vasopressin tannate (Pitressin),[16] ACTH,[12] cortisone,[12] thyroxine,[15] or parathyroid extract[17] fails to restore the ability to develop hypertension with a DCA treatment in the hypophysectomized animal. Recently this deficiency was partially overcome by treating hypophysectomized rats with lyophilized whole rat pituitary (LWP).[18] Under these conditions DCA produced hypertension and slight cardiorenal lesions. This was interpreted as a confirmation of the hypothesis that a substance or combination of substances in whole pituitary, not present in single fractions previously tested, is an essential cofactor in the production of hypertension by DCA.[18] "Mineralocorticoid hypertension, therefore, would appear to result from the interplay of three factors: the hormone itself; an adequate supply of dietary sodium; and a source of a pituitary fraction, nature as yet unidentified."[18]

The pituitary has a profound influence on established DCA hypertension. Removal of the pituitary from rats with established DCA hypertension[4, 12, 19] was followed by a fall in blood pressure and regression of arterial and renal lesions.

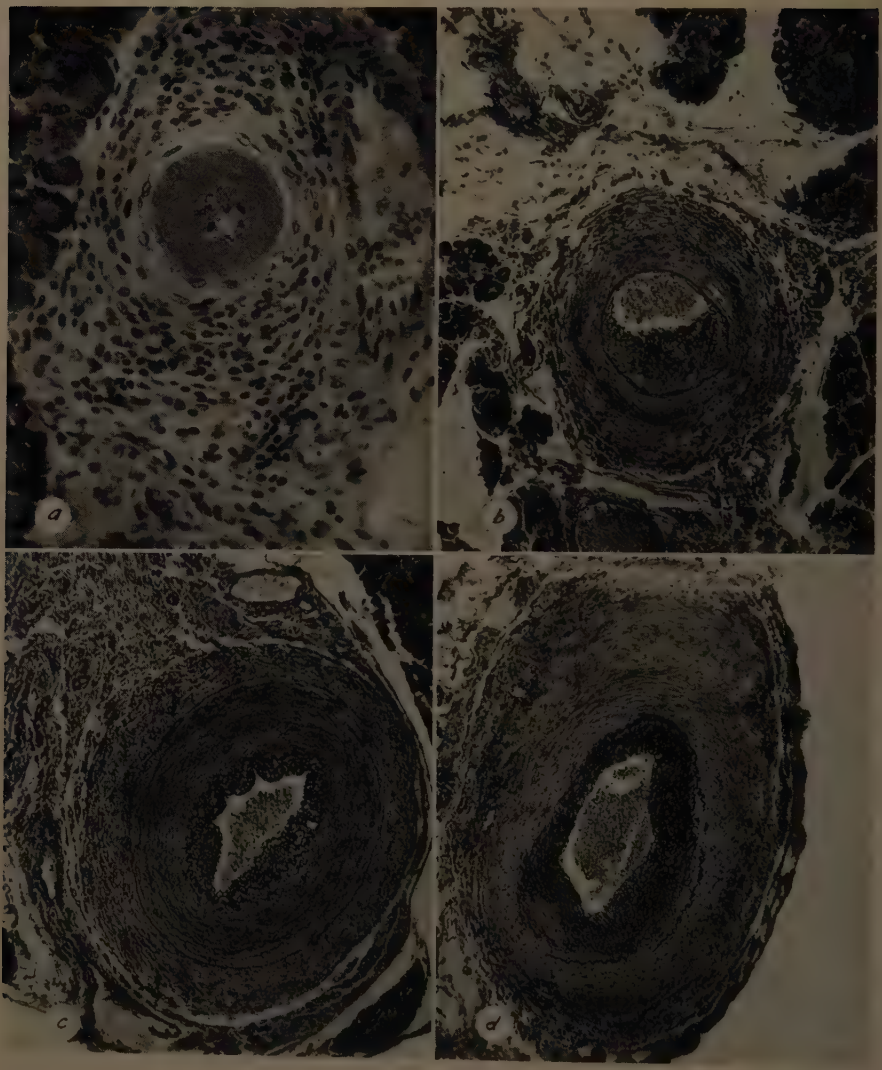

FIGURE 3. Pancreatic vessels of the same rat: (*a*) acute form of periarteritis nodosa (hematoxylin-eosin stain, high magnification); (*b*) small arteriole, showing an eccentric intimal proliferation (Weigert's stain, medium magnification); (*c* and *d*) healed lesions, showing scarring of the media and thickened elastica. Weigert's stain, low magnification.

Influence of the Thyroid

Thyroidectomy in dogs does not prevent or cure renal hypertension.[8, 20, 21, 22] In the rat, thiouracil administration reduces the elevated blood pressure of rats with renal hypertension.[23] It was shown that interference with the function of

TABLE 4

INFLUENCE OF THYROPARATHYROIDECTOMY ON TISSUE LESIONS OF DCA-TREATED RATS*

Lesions in:	Controls		DCA†		DCA† + Thyroparathyroidectomy‡	
	Incidence	Severity	Incidence	Severity	Incidence	Severity
	percentages					
Brain	0	0	60	72	0	0
Heart	0	0	90	78	0	0
Kidney	0	0	90	78	0	0
Mesenteric arteries	0	0	70	75	77	92

* The animals were not unilaterally nephrectomized, drank 1 per cent NaCl, and were sacrificed at week 10.

† Three 25-mg. pellets.

‡ On day −3.

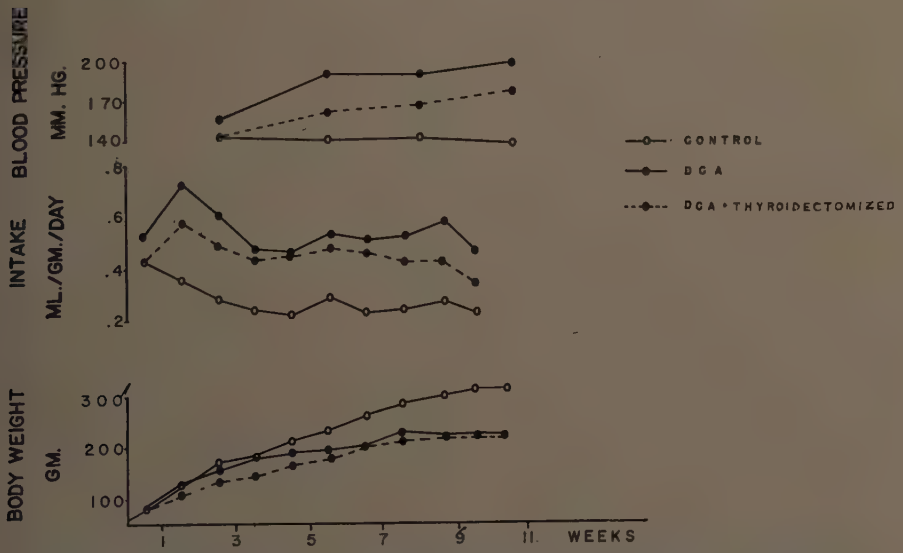

FIGURE 4. Changes in blood pressure, fluid intake, and body weight of rats receiving DCA. For details see TABLE 4.

the thyroid by surgery (thyroidectomy) or chemical means (propylthiouracil administration) had a profound effect on the DCA-induced syndrome.[24]

Thyroidectomy prior to DCA implantation inhibited hypertensive renal and cardiac lesions and enhanced the periarteritis nodosa. Cardiac and renal enlargement was diminished, but still present. Mortality was diminished. Thiouracil administration, when started simultaneously with DCA implantation, had similar effects except that no periarteritic lesions were present. These conclusions apply to other kinds of hormonal hypertension produced by STH,[25] MAD,[26] and 9α-chlorocortisol.[27]

The original experiments mentioned above were of short duration, and the

possibility remained that the development of hypertension was not prevented but delayed and would have become manifest had the period of observation been longer. This problem was clarified in an experiment in which: "The effect of the thyroid on the development of DCA hypertension was studied by comparing the behavior of DCA-treated thyroparathyroidectomized rats with DCA-treated controls. Thyroparathyroidectomy prior to implantation of a single 20 mg. DCA pellet prevented hypertension and all tissue lesions except periarteritis nodosa during a 15-week period in which the animals slowly matured. Subsequent administration of minimal doses of thyroxine (insufficient to restore the histological picture of the hypophysis completely to normal) was associated with the development of hypertension and cardiac hypertrophy although the growth rate was not materially accelerated."[17] It was concluded that the thyroid hormone may represent a significant factor in the development of DCA hypertension. The ability of DCA to induce periarteritis nodosa without hypertension in the thyroparathyroidectomized rat suggests that this preparation may offer a new approach to studies of the pathogenesis of periarteritis.[17]

To study further the influence of the amount of DCA, the length of the treatment and of thyroxine administration, we performed 3 experiments. Details and results are in TABLES 1, 3, and 4 and in FIGURES 1 and 4. These experiments show that, in the absence of the thyroid, moderate doses of DCA fail to produce hypertension. When high doses are used, hypertension appears but to a lesser degree than in the intact controls. The lesions in the kidney, heart, and brain are absent or considerably diminished, and again the arterial lesions in the mesenteric vessels are aggravated. In the presence of physiological doses of thyroxine, thyroidectomized rats reacted to DCA, as did the intact controls.

In most of the experiments mentioned above surgical parathyroidectomies were performed; that is, the parathyroid gland was also removed. However, we believe that the results are attributable to the thyroidectomy and not to the absence of the parathyroid. Our belief is based mainly on the propylthiouracil and thyroxine experiments, but also on the indirect evidence afforded by experiments in which overdosage with thyroxine aggravated[4, 28, 29, 30] the lesions produced by DCA.

Thyroparathyroidectomy or treatment with propylthiouracil of rats with meta-DCA hypertension was followed by a moderate fall in blood pressure that reached its minimum at the end of the first week and then reverted toward pretreatment levels despite continuation of treatment.[4] Removal of the thyroid lowered the blood pressure in rats treated with DCA for 19 days, but not in rats treated for 33 days,[24] which probably indicates that thyroidectomy is effective only up to the moment at which a status of a permanent hypertension has been reached.

Summary and Conclusions

It is well known that the administration of desoxycorticosterone acetate (DCA) to rats under suitable conditions produces a syndrome of hypertension, periarteritis, and cardiorenal lesions.

If performed prior to DCA administration, hypophysectomy prevents completely the development of the syndrome. Even in rats with established DCA-induced cardiovascular disease the removal of the hypophysis is able to reverse the hypertension and improve the tissue lesions.

Removal of the thyroid prior to DCA administration prevents hypertension and all pathological lesions except periarteritis nodosa.

Hypophysectomy and thyroidectomy have similar effects in experimental hypertensive disease induced with hormones other than DCA. These results are discussed.

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CARDIOVASCULAR CHANGES INDUCED IN CHOLINE-DEFICIENT RATS BY GROWTH HORMONE*

George F. Wilgram

Banting and Best Department of Medical Research, University of Toronto, Toronto, Canada

Fatty livers[1] and hemorrhagic kidneys[2] resulting from dietary choline deficiency were shown to occur when Hartroft and his colleagues in 1952 described aortic lesions in rats kept on hypolipotropic diets for many months.[3] Somewhat later these findings were extended in our laboratory to changes occurring in the acute state of severe choline deficiency.[4] Further work was undertaken to elucidate the etiology, pathogenesis, and biochemistry of cardiovascular lesions in choline deficiency.[5]

In the course of this endeavor we found that injections of growth hormone accentuate and aggravate cardiovascular lesions in choline-deficient rats.[6] As such, the finding is of only academic interest and has no practical application. However, I present it here for two reasons: first, the administration of this hormone influences the metabolism of the whole organism, thereby affecting the anabolism and catabolism of the cardiovascular system; second, the experience and knowledge gained in the pursuit of this work yielded information that enabled us to develop, by other methods, cardiovascular lesions in rats that closely resembled human heart disease.

Histopathology

Young rats on choline-deficient diets may develop the following cardiovascular lesions after 3 weeks in acute experiments or after approximately 5 months in long-term trials.

First, a focal cardionecrosis preceded by the appearance of stainable lipid within the muscle fibers. Polymorphonuclear leukocytes and, later, lymphocytes appear at the site of lipid-laden muscle fibers. Interstitial edema is frequently pronounced. Sometimes death of the animal occurs before this interstitial myocarditis can develop further into frank focal necrotic areas. If the animal survives, the inflammatory and necrotic debris is absorbed and removed, leaving focal fibrotic scarred tissue in the myocardium. Thus far, no definite topographical relation between the coronary tree and these focal necrotic areas has been discerned.

Second, stainable lipid is seen in the media of coronary arteries. Intracellular and extracellular lipids also may be observed in the intima and the adventitia, but the location of main accumulation is the media. Sometimes this stage of lipid appearance is followed by signs of necrosis in the components of the media.

Third, the same picture as in the coronaries is observed in the aorta of choline-deficient rats, except that medial necrosis is much more pronounced

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and much more common. This medial necrosis is then followed by calcification and, frequently, the aorta is totally rigid, hardened, widened, and has the appearance of a bamboo stick. The intima of the aorta is but rarely involved and on occasion shows only a "hyaline cap" over areas that are necrotic and calcified in the underlying media. This hyaline cap is apparently a proliferative connective tissue response to the injury of the medial structures beneath them. The elastic elements of the aorta are shriveled and broken. Microscopically, the whole media looks like a condensed calcified bar. On occasion this barlike tissue breaks, leading to a picture that has been termed collar-button fracture by Hartroft.[3] The whole syndrome may therefore most favorably be compared with Mönckeberg's sclerosis in human arteries. There is no similarity with the process of atherosclerosis as encountered in man.

Biochemistry

Choline-deficient animals always exhibit blood lipid values that are lower than their choline-supplemented controls. This holds true whether the blood lipids are expressed physicochemically as lipoproteins or biochemically as cholesterol, phospholipids, and neutral fat. Contrasted with the low blood values are the levels of liver lipids in choline-deficient animals which, of course, are always elevated as compared with their choline-supplemented controls.

Effect of Growth Hormone

The lesions described above developed in rats on diets that were severely deficient in lipotropic factors. It is possible, however, to choose the experimental conditions in such a way that the animals are in a state of borderline deficiency (TABLE 1). In this borderline state of deficiency the animals have fatty livers and lowered blood lipids, but no renal and no cardiovascular changes. This borderline state can be achieved by various means: for example, by changing

TABLE 1
COMPOSITION OF CHOLINE DEFICIENT DIET WCA*

Casein	7
Peanut meal†	28
Soya protein‡	5
Salt mixture[7]	3
Sucrose vitamin mixture[7]	1
Sucrose	11
Starch	10
Lard	34
α-Tocopherol acetate§	0.015
Cod-liver oil‖	0.01
Corn oil§	1

* This diet is borderline deficient in lipotropic factors at a room temperature of 72° F. for female white rats of 110 gm. average weight.

† Extracted with 50, 75, and 95 per cent ethanol.

‡ Water-washed "alpha proteins" (Glidden Co., Baltimore, Md.).

§ Fat-soluble vitamins dissolved in corn oil so that 1 per cent of dietary corn oil will supply the desired amount of fat-soluble vitamins.

‖ Obtained from Ayerst McKenna and Harrison, Montreal, Canada; contains 200,000 I.U. vitamin A and 50,000 I.U vitamin D/gm.

TABLE 2

CARDIOVASCULAR CHANGES IN CHOLINE-DEFICIENT RATS INDUCED BY GROWTH HORMONE

Group	Av. wt. (gm.)	Av. food intake (gm./day)	No. of rats	Pathology					Total	Per cent with CV lesions
				Kidney			CV system			
				Frosting	HKL	Total	Cardiac	Aortic		
Control*	112	14.5	18	6	0	6/18	3	0	3/18	16
Growth hormone	112	14.3	7	3	4	7/7	4	3	7/7	100
Testosterone	110	11	9	3	6	9/9	6	3	6/9	66
Growth hormone + testosterone	117	12.6	10	3	7	10/10	5	4	5/10	50

* Female white rats on choline-deficient diet WCA. All these animals have fatty livers and low blood lipids.

the methionine content of the diet, or by using older animals or female rats that are less susceptible to the adverse effects of choline deficiency than are males. If purified growth hormone is administered under such conditions, the severity of choline deficiency is greatly aggravated; this can be seen from TABLE 2. One may observe that with the administration of growth hormone, bilateral renal hemorrhagic cortical necrosis sets in, and the death of the animal ensues. With the appearance of kidney damage, cardiovascular lesions are also demonstrable in corresponding degrees. The control animals had the basic signs of choline deficiency: namely, a fatty liver and lowered blood lipids. However, the severe degrees of choline deficiency, that is, kidney damage and cardiovascular lesions, were observed only when growth hormone was administered to experimental animals that were pair-fed with their saline-injected controls. This clearly demonstrates that the aggravation of choline deficiency and the appearance of cardiovascular lesions depended under these conditions on the injection of growth hormone. This experiment also indicated that the etiology of cardiovascular lesions in choline deficiency depends more on the induction of kidney damage than on choline deficiency per se.

Interpretation of the Action of Growth Hormone

This experiment should not be viewed exclusively from the aspect of cardiovascular disease, but also from the point of the effect of growth hormone on general metabolism. Growth hormone and other protein anabolic hormones such as testosterone favor nitrogen retention and protein synthesis. Methionine is one of the amino acids necessary for protein synthesis. However, methionine may also serve as a precursor for the biosynthesis of choline. If methionine is in short supply it will be used for purposes of growth in preference to biosynthesis of choline.[8-11] If growth and protein synthesis are stimulated and the demand for methionine is heavy, little of it will be available for the biosynthesis of choline. This means that the state of choline deficiency would be aggravated by stimulation of growth, as less choline would be synthesized from methionine, its biochemical precursor.

The actual findings may thus be summarized as follows. Rats on a diet that is on the border line of deficiency in methionine and free from choline will develop fatty livers and lowered blood lipids. If such animals are treated with growth hormone, the state of choline deficiency is aggravated and, as a consequence, renal and cardiovascular lesions appear.

Clinical Applications

Evidence that the cardiovascular lesions observed in choline deficiency are secondary to renal injury is strong, but still only circumstantial. Other authors, however, have different opinions. Kleinerman[12] attributes these lesions to a deficiency of methionine, while Hartroft[13] contends that a lack of dietary choline per se is the primary cause of these changes. Hartroft also suggests that the etiology of cardiac and coronary changes in choline deficiency is not the same as that of aortic sclerosis, and he proposes that these two groups of vascular changes should not be grouped together under one heading. Space does not permit further elaboration of this subject here.

FIGURE 1. Cardiac infarct and complete coronary occlusion induced experimentally in the rat by means of hyperlipemia and vascular injury. A medium-sized coronary artery with 2 of its branches, cut in cross section. Extensive atherosclerosis appears in all 3 lumina, occluding 1 of them completely. The adjacent cardiac muscle exhibits scattered areas of necrosis in the lower half of the photograph, while a large area of frank necrosis can be seen in the upper portion. This infarct is well circumscribed, wedge-shaped, and has a classic zone of leukocytic infiltration around it. Low power, frozen section, oil red O stain. Fat appears black.

It became obvious, however, that these cardiac, coronary, and aortic lesions have little if any resemblance to atherosclerosis in man. Histopathology, biochemistry, etiology, and pathogenesis of the Mönckeberg type of vascular disease as observed in choline deficiency differ essentially from atherosclerosis as seen in man. Human cases suffering from Mönckeberg's sclerosis of the peripheral arteries on the other hand do not appear to be choline-deficient. Consequently, no clinical applications were possible.

Development of Knowledge about the Induction of Coronary Infarcts

A thorough search of the literature dealing with the rat as an experimental animal for the induction of atherosclerosis and cardiac infarcts revealed that this animal was believed to be resistant to the induction of atherosclerosis for many decades.[14] However, there were some encouraging reports in the literature and, aided by our own experience with cardiovascular lesions in choline deficiency, we set out to develop a reliable dietary method for the induction of atherosclerosis, coronary occlusion, and cardiac infarcts in rats. This method entailed an experimental induction of hyperlipemia in combination with vascular injury. A basal diet rich in fat and dried egg yolk was supplemented with extra cholesterol, sodium choleate, thiouracil, and vitamin

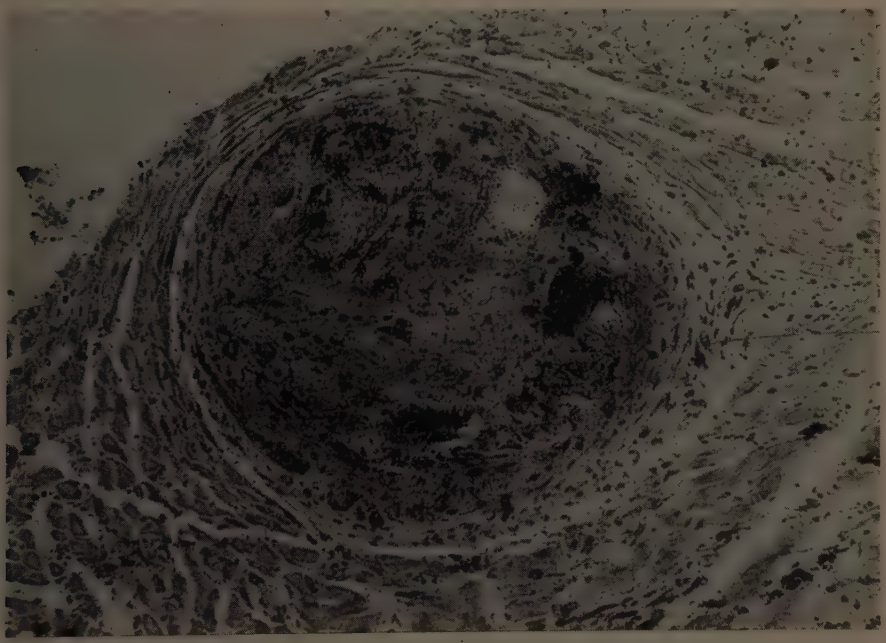

FIGURE 2. A subepicardial coronary artery, completely occluded by atheromatous and thrombotic masses. Granulation tissue invades the lumen toward the center. Thrombotic material is intermingled with fat, debris, cholesterol crystals, proliferating cells, and increased connective tissue ground substance. High power, frozen section, oil red O stain. Fat appears black.

D. This regimen is rich in choline and has no resemblance to the choline-deficient diet already cited. This experimental approach to the induction of coronary infarcts is mentioned in this presentation only, because the experience gained in the performance of experiments with choline-deficient diets led to the knowledge of conditions that are necessary for the experimental induction of atherosclerosis. The method developed on the basis of this knowledge had yielded reproducible results in many different experimental trials. In our final experiment on 27 rats weighing initially 450 gm. we observed the following results after an average period of 8 months: coronary infarcts, 14 per cent; coronary occlusion, 33 per cent; coronary atherosclerosis, 63 per cent; coronary atheromatous and lipomatous lesions, 88 per cent. FIGURES 1 and 2 illustrate one of the infarcts observed.

Summary

Injections of growth hormone increase the demand for methionine in a choline-deficient diet and thereby aggravate the state of choline deficiency in rats. This aggravation of a lack of choline leads to the development of bilateral renal cortical necrosis, which is presumably responsible for the concomitant induction of cardiac, coronary, and aortic changes in choline-deficient rats.

These findings in choline-deficient rats resemble the Mönckeberg type of vascular disease and have no direct or immediate clinical application. They were very useful, however, in providing us with the know-how for the development of other methods to induce cardiovascular changes in rats. These newly developed methods yielded lesions that are indeed an appropriate experimental counterpart to atherosclerotic heart disease in man.

This demonstrates again that research that had no apparent application yesterday may prove to be of practical value today.

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INFLUENCE OF CORTISONE ON LIPID DISTRIBUTION AND ATHEROGENESIS

Abraham Dury

Dorn Laboratory for Medical Research of the Glendorn Foundation, Bradford Hospital, Bradford, Pa.

Administering large doses of cortisone[1-7] daily to cholesterol-fed rabbits or rendering them alloxan-diabetic[8, 9] inhibits the expected development of atherosclerosis. Injections of Tween 80 and Triton A20[10, 11] also protect cholesterol-fed rabbits from developing these vascular lesions. Since failure to develop atherosclerosis occurred in the presence of serum cholesterol levels not appreciably different from those of untreated rabbits, these observations are particularly relevant to the problem of the pathogenesis of atherosclerosis. The results suggest that development of experimental atherosclerosis is not dependent upon the mere existence in the circulating blood of high levels of cholesterol, but on other factor(s) as yet undetermined. Since lipid deposition is essential for the experimental development of atherosclerosis, the inhibitory influence of cortisone or Triton administration, as well as of alloxan diabetes, must be implemented by interference with the deposit of lipids in the arterial wall.

In this report we shall present some already published and unpublished data of the alterations in blood lipids and their physicochemical state that coexist in rabbits treated with cortisone. Information of this kind might elucidate the mechanism(s) by which cortisone inhibited the expected development of atherosclerosis.

Experiments on Rabbits Fed a Normal Diet

A few remarks on the cortisone-treated, normally fed rabbit are warranted, since they will illustrate the type of alterations in lipid distribution and metabolism to be expected following administration of different amounts of cortisone for different periods of time.

Daily administration of 10 mg. of cortisone to normally fed rabbits for 14 consecutive days caused considerable changes in plasma and liver lipid distribution (TABLE 1). In both tissues phospholipid and neutral fat were greatly increased, and significantly increased concentrations of free or esterified cholesterol also were determined in plasma and liver. Furthermore, this amount of cortisone caused an increase in liver weight to threefold that of the normal animal. Our studies with radioactive phosphorus (P^{32}) also revealed a greatly accelerated synthesis of liver and plasma phospholipid in rabbits submitted to this cortisone regime. It may be noted here that these alterations in lipid distribution and phospholipid metabolism are similar to those determined in cholesterol-fed rabbits administered 10 mg. of cortisone daily over a period of 3 to 4 months' duration.

TABLE 2 shows the effect on plasma and liver lipid distribution of 5 mg. of cortisone administered 3 times a week for 100 days. In these animals neither weight of the liver nor its lipid content are different from controls. The hyper-

TABLE 1

EFFECT OF BRIEF-PERIOD, LARGE-DOSE CORTISONE ADMINISTRATION ON LIPID DISTRIBUTION IN RABBITS ON A NORMAL DIET

Groups	Total lipid	Phospholipid	Cholesterol			Neutral fat	
			Total	Free	Ester		
Plasma lipid fractions (mg./100 ml.)							
Controls	448	158	105	31	74	130	
Cortisone*	1225†	246†	122	66†	56	845†	
Liver lipid fractions (gm./total liver)							
							Wt. liver (gm.)
Controls	4.1	3.6	0.20	0.18	0.02	0.4	80.5
Cortisone	13.5†	4.4†	0.46†	0.32†	0.14†	8.5†	297.0†

* Cortisone, I.M., 10 mg. each day for 14 consecutive days.
† Significantly different from controls.

TABLE 2

EFFECT OF EXTENDED CORTISONE ADMINISTRATION ON LIPID DISTRIBUTION IN RABBITS ON A NORMAL DIET

	Total lipid	Phospholipid	Cholesterol			Neutral fat	
			Total	Free	Ester		
Plasma lipid fractions (mg./100 ml.)							
Controls	397	121	76	23	50	164	
Cortisone*	1070†	194†	75	46†	28†	779†	
Liver lipid fractions (gm./total liver)							
							Wt. liver (gm.)
Controls	4.2	3.1	0.31	0.27	0.04	0.8	97.2
Cortisone	4.5	2.9	0.28	0.24	0.04	1.2	114.5

* Cortisone, I.M., 5 mg., 3 times/week over 100 days.
† Significantly different from controls.

lipidemia present in the treated rabbits is also the resultant of increased levels of neutral fat, phospholipid, and free cholesterol, as was the case with a large dose for a short period.

FIGURES 1 and 2 illustrate in another way the nature of the altered lipid distribution of plasma and liver found in these two studies. In FIGURE 1 the plasma total lipid increment of cortisone-treated rabbits is shown as percentage of their controls. The other bars represent the relative contribution of phospholipid, free and ester cholesterol, and neutral fat, respectively, to the total lipid increment. Presentation of the data in this manner clearly shows that: (1) the percentage increase of total lipid over respective controls was the same

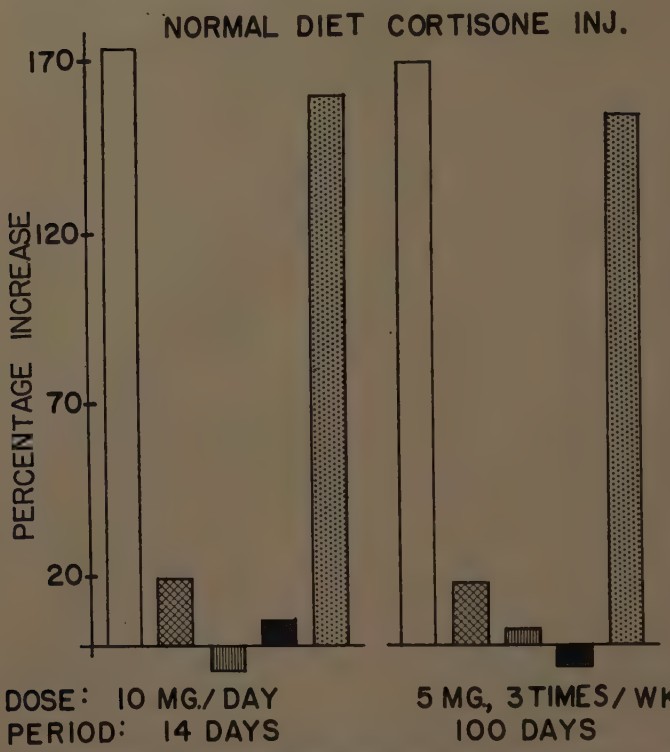

FIGURE 1. The pattern of alteration of lipid distribution in the plasma of cortisone-treated rabbits over controls fed a normal diet, showing the relative contribution of lipids to the total increase in plasma. See text for description.

with both kinds of cortisone regimes; (2) neutral fat is the paramount contributor to the total lipid increment; and (3) the relative contributions of phospholipid and cholesterol to the existing hyperlipidemia are very small, indeed. In a similar fashion, FIGURE 2 illustrates the relative quantities contributed by the different lipids to the increment in liver total lipid. The findings in the liver are different with the 2 cortisone regimes. In rabbits that received 10 mg. of cortisone daily for 14 days an elevation of neutral fat is primarily responsible for the increase liver lipid, while in rabbits administered 5 mg. of cortisone 3 times weekly for 100 days the neutral fat was only slightly elevated compared to controls, and liver total lipid was not significantly different from normal. Evidently, hyperlipidemia due to marked increments of neutral fat in the circulating blood followed administration of rabbits with large doses of cortisone for a short period or the reverse, but excessive mobilization of liver neutral fat occurred only when large doses of cortisone were administered.

The data presented in TABLE 3 indicate that a disturbance in the lipid-transporting mechanism may be involved in the accumulation of neutral fat in

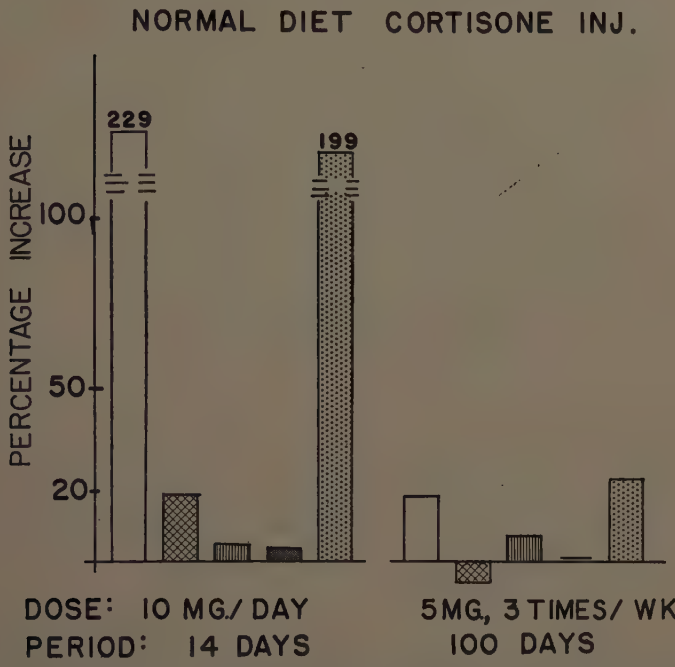

FIGURE 2. The pattern of alteration of lipid distribution in the liver of cortisone-treated rabbits over controls fed a normal diet. Note total increase in liver. See text for description.

TABLE 3

EFFECT OF EXTENDED CORTISONE ADMINISTRATION ON THE RELATIONSHIP OF LOW-DENSITY LIPOPROTEIN CLASSES IN NORMALLY FED RABBITS

Groups	Low-density lipoproteins (mg. per cent)	
	S_f 0 to 12	S_f 12 to 400
Controls (12)	195* (68 to 276)	28 (0 to 102)
Cortisone† (6)	57 (0 to 90)	216 (117 to 414)

* Mean and range of values.
† Cortisone, I.M., 5 mg., 3 times/week for 100 days.

blood. In rabbits administered 15 mg. of cortisone weekly during a period of 100 days the quantities of low-density lipoproteins of the S_f 0 to 12 and S_f 12 to 400 classes are inverted compared to those of controls. It is also notable that the concentration of lipoproteins of the S_f 0 to 12 class in the treated group is significantly less than in the controls. These findings are in accord with those of Pierce and Bloom[12] and of Pierce[13] of a "block" caused by cortisone administration in the conversion of high S_f lipoproteins to those of the lower classes.

TABLE 4

EFFECT OF DAILY CORTISONE ADMINISTRATION CONCURRENT WITH CHOLESTEROL SUPPLEMENTATION OF DIET ON SEVERITY OF AORTIC ATHEROSCLEROSIS IN RABBITS

Groups	No. of aortas graded with lesions of:					Incidence ratio	Severity factor*
	0	1+	2+	3+	4+		
Saline (13)	1	2	0	6	4	12/13	273
Cortisone (13)	9	3	1	0	0	4/13	12

Regular rabbit pellet-food diet supplemented with 1 gm. cholesterol daily for 4 months. Cortisone, 2.5 mg., was injected I.M. each day except Sundays.

* Average degree of severity × percentage of incidence of lesions in the group.

Experiments on Cholesterol-Fed Rabbits

On the basis of these studies the development of aortic atherosclerosis and the coexisting alterations in plasma lipid distribution and their physicochemical state were investigated in cholesterol-fed rabbits administered daily a small amount of cortisone. Moreover, it had not yet been shown that adherence to this type of cholesterol and cortisone regime inhibits the development of atherosclerosis.

The data in TABLE 4 show that development of aortic atherosclerosis is greatly inhibited in cholesterol-fed rabbits administered 2.5 mg. of cortisone daily for 4 months. Whether the severity factor is used as an index or the number of animals with aortic lesions visually graded 3 plus and 4 plus, it is equally evident that the cortisone regime caused an interference with the deposition of lipids in the aortic intima of these animals. This is particularly remarkable, since plasma cholesterol concentrations of untreated and cortisone-administered rabbits were not appreciably different (TABLE 5). It may be seen that only plasma-neutral fat and phospholipid levels were significantly increased in cortisone-injected rabbits. As for the liver, its size and lipid fractions are not different from controls, excepting a significantly increased content of neutral fat.

The altered quantitative relationships of plasma lipid fractions of cholesterol-fed rabbits administered cortisone or untreated over the normal are depicted in FIGURE 3. The bars show, respectively, the increment of total lipid as a percentage of the total lipid of the normally-fed group and the relative contributions of phospholipid, free and ester cholesterol, and neutral fat to the total lipid increase. It is evident that an increment in ester cholesterol is primarily responsible for the hyperlipidemia present in cholesterol-fed rabbits. Phospholipid and free cholesterol also contribute significant amounts to the total lipid change from normal value, but neutral fat has not contributed to the hyperlipidemia extant in untreated cholesterol-fed rabbits. On the other hand, in cholesterol-fed cortisone-injected rabbits the hyperlipidemia is due to the greatly increased amount of neutral fat and esterified cholesterol and a smaller contribution by phospholipid.

In a similar manner FIGURE 4 shows the relative contributions of the plasma-

TABLE 5

EFFECT OF DAILY CORTISONE ADMINISTRATION CONCURRENT WITH CHOLESTEROL SUPPLEMENTATION OF DIET ON LIPID DISTRIBUTION

Groups	Total lipid	Phospho-lipid	Cholesterol			Neutral fat	
			Total	Free	Ester		
Serum lipid distribution (mg./100 ml.)							
Saline	1935	391	894	236	658	195	
Cortisone	2755*	617*	732	267	465	1089*	
Liver lipid distribution (gm./total liver)							
							Wt. liver (gm)
Saline	7.8	4.5	1.8	0.7	1.3	0.4	136.4 (106 to 175)
Cortisone	8.9	4.0	1.4	0.4	1.1	2.8*	159.0 (107 to 196)

Regular diet supplemented with 1 gm. cholesterol daily for 4 months. Cortisone, 2.5 mg., I.M. daily except Sundays.

* Significantly different from controls.

FIGURE 3. The pattern of alteration of lipid distribution in the plasma of untreated rabbits and cortisone-treated, cholesterol-fed rabbits over untreated rabbits on a normal diet. See text for description.

lipid fractions responsible for the difference in total lipid between cortisone-injected and untreated cholesterol-fed rabbits. It is evident that the difference between the two groups is the result of a greatly increased level of neutral fat only in the rabbits administered cortisone.

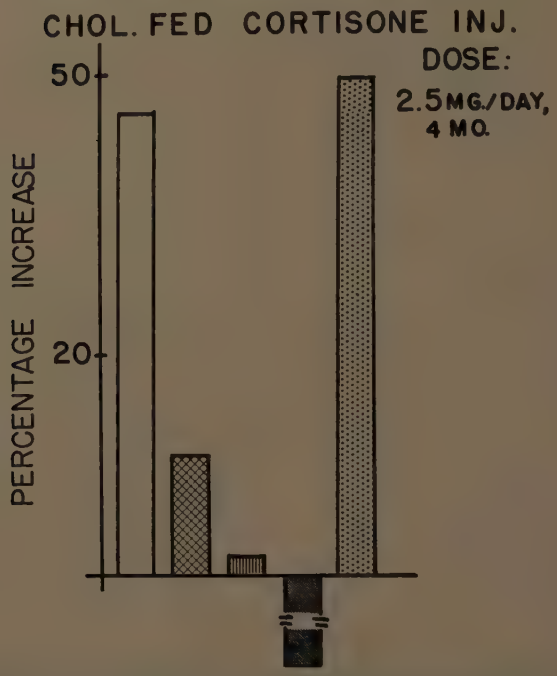

FIGURE 4. The pattern of alteration of lipid distribution in plasma of cortisone-treated, cholesterol-fed rabbits over untreated, cholesterol-fed animals. See text for description.

TABLE 6

EFFECT OF CORTISONE ON LIPOPROTEINS OF CIRCULATING BLOOD OF CHOLESTEROL-FED RABBITS

Treatment	Low-density lipoproteins (mg./100 ml.)		Serum lipids	
	S_f 0 to 12	S_f 12 to 400	Total cholesterol (mg. per cent)	Neutral fat (mg. per cent)
Normal diet				
Saline	124 (69 to 204)	80 (11 to 311)	69 (40 to 102)	164 (77 to 262)
Cholesterol-supplemented (1 gm./day for 4 months)				
Saline	558 (336 to 838)	721 (186 to 2187)	894 (582 to 1260)	195 (139 to 346)
Cortisone, 2.5 mg/day*	851 (620 to 1404)	2359 (1149 to 5703)	732 (518 to 1250)	1089 (654 to 2417)

* Except Sundays.

The amounts of S_f 0 to 12 and S_f 12 to 400 lipoproteins present in the blood of normally fed rabbits was discussed. The effect of cortisone on circulating levels of low-density lipoproteins in cholesterol-fed rabbits is shown in TABLE 6. When a cholesterol-supplemented diet is fed, rabbits exhibit a marked eleva-

tion of the lipoproteins of S_f 0 to 12 and S_f 12 to 400 classes. The increase in the former class is believed to be associated with the large increment of cholesterol and its transporting system in the plasma, while the concurrently elevated quantity of S_f 12 to 400 lipoproteins is attributed to a "spilling-over" process,[14] as yet unclear, from the accumulated lower S_f classes. In cholesterol-fed, cortisone-injected rabbits the lipoproteins of S_f 0 to 12 show a small but significantly increased level, while the amount of lipoproteins of S_f 12 to 400 is threefold that of the untreated group. The latter is not unexpected,[15, 16] since cortisone administration results in a marked increase in plasma-neutral fat. The findings are in accord with the evidence[15] that lipoproteins of the high S_f classes in general have less cholesterol and more neutral fat per molecule than those of the lower S_f classes (S_f 80). It is probable that the increase of S_f 12 to 400 lipoproteins in the circulating blood of cortisone-administered rabbits is superimposed on that amount of this class present as a result of the cholesterol supplementation of the diet alone.

Pierce and Bloom[12] reported evidence in normally fed rabbits injected with cortisone for a short period suggestive of a "metabolic block" in the conversion of high S_f lipoproteins to classes below S_f 80. Recent studies[17, 18] have shown that the clearing factor (lipoprotein lipase) is a physiologically active enzyme necessary for the hydrolysis of the triglyceride of lipoproteins and the removal of neutral fat from the blood. Theoretically, a defect in the production, a release, or an inhibition of clearing factor activity might account for the findings in cortisone-treated, cholesterol-fed rabbits. Accordingly, measurements were done of clearing activity *in vitro*,[19, 20] using plasmas of untreated and cortisone-injected, cholesterol-fed rabbits, mixed with postheparin active serum and a standard fat emulsion and incubated at 30° C. for 60 min. It may be seen (TABLE 7) that the clearing of a standard fat emulsion in mixtures containing plasma of cortisone-injected rabbits is 60 per cent of the value determined

TABLE 7

EFFECT OF PREVIOUS INCUBATION OF PLASMAS OF UNTREATED AND CORTISONE-TREATED CHOLESTEROL-FED RABBITS WITH POSTHEPARIN PLASMA AND WITH SUBSTRATE ON LIPOPROTEIN LIPASE ACTIVITY

Fall OD (per cent)		Difference (per cent)
Controls	Cortisone*	
Test plasma + postheparin plasma†		
55 ± 2.7	33 ± 1.3	60
Test plasma + substrate‡		
59 ± 3.2	34 ± 3.7	57

* Cortisone, 2.5 mg./day except Sundays for 4 months.

† Incubation mixture contained: 0.5 ml. "test" rabbit plasma, 0.2 ml. phosphate buffer, 0.2 ml. postheparin plasma; incubated at 30° C. for 20 min.

‡ Incubation mixture contained: 0.5 ml. of "test" rabbit plasma, 0.2 ml. buffer, 0.1 ml. of coconut oil emulsion; postheparin plasma and albumin added after 20 min. incubation. All values are average fall OD after 60 more min. of incubation following addition of other components of reaction mixture.

TABLE 8

EFFECT OF HIGH CHOLESTEROL DIET ALONE AND WITH CORTISONE ADMINISTRATION ON DISTRIBUTION OF SERUM LIPID OF RABBITS

Experimental groups	Total lipid	Phospho-lipid	Cholesterol			Neutral fat
			Total	Free	Ester	
Differences between pre-experimental and experimental values (mg./100 ml.)						
Cholesterol-fed only						
14 days	+859*	+188*	+437*	+278*	+160*	+126*
30 days	+1407*	+280*	+662*	+234*	+433*	+168*
Diff. P =	0.02	0.02	0.05	ns	0.01	ns
Cholesterol-fed + cortisone†						
14 days	+649*	+125*	+148*	+138*	+16	+398*
30 days	+2295*	+395*	+294*	+134*	+160*	+1453*
Diff. P =	<0.01	<0.01	0.03	ns	0.01	0.01

* Significantly different from pre-experimental average value.
† Cortisone, I.M., 3.0 mg/day except Sunday.

with plasmas of the untreated group. The results of the 2 kinds of clearing tests indicate it is improbable that a primary interaction between the substrate and plasma of cortisone-treated rabbits could account for the inhibition of clearing observed in mixtures containing the latter plasma. In that case little or no clearing would occur after adding the postheparin plasma.

It appeared that similar studies during a period before atherosclerosis usually develops might provide insight into the *modus operandi* of cortisone, which results in the retarded development of atherosclerosis. Experiments, therefore, were done to analyze the effect of cortisone and cholesterol feeding on the basis of a longitudinal study.

TABLE 8 shows the differences between pre-experimental values of plasma lipids and those determined at 14 and 30 days in rabbits on a high-cholesterol diet alone and in conjunction with cortisone administration. Except for ester cholesterol in the cortisone-treated group at 14 days, there is a significantly increased quantity of each lipid fraction in rabbits of both groups. Between 14 and 30 days both groups presented an additional increment of plasma phospholipid, ester, and total cholesterol. However, neutral fat is increased further only in those receiving cortisone.

Although the alterations in plasma lipid distribution from pre-experimental levels appear to be parallel in both groups, the administration of cortisone resulted in differences in lipid fractions during the experiment (TABLE 9). At the end of two weeks the group administered cortisone had levels of total, free, and esterified cholesterol significantly lower, while neutral fat was significantly higher than that present in untreated cholesterol-fed rabbits. After one month these differences between cortisone and untreated rabbits still exist but, notably, the amounts of neutral fat and phospholipid are increased considerably in the former group. It appears that cortisone exerted a restraining influence on the development of a high level of esterified cholesterol in the blood due to ingestion of a cholesterol-supplemented diet alone and also induced a large increment of neutral fat and some increase of phospholipid.

TABLE 9

EFFECT OF CORTISONE ON SERUM LIPID FRACTIONS OF CHOLESTEROL-FED RABBITS

Groups	Total lipid	Phospho-lipid	Cholesterol			Neutral fat
			Total	Free	Ester	
Difference (mg./100 ml.) of lipid fractions between groups:						
Saline vs. cortisone*						
14 days	−179	−58	−297	−139	−152	+311
P =	ns	ns	<0.01	0.02	<0.01	0.02
30 days	+919	+120	−376	−99	−281	+1324
P =	ns	0.03	0.01	ns	0.01	0.01

* Cortisone, I.M., 3.0 mg. each day except Sunday.

TABLE 10

EFFECT OF CHOLESTEROL FEEDING ALONE AND IN COMBINATION WITH CORTISONE ADMINISTRATION ON THE DIFFERENCE BETWEEN PRE-EXPERIMENTAL AND FINAL VALUES OF LOW-DENSITY LIPOPROTEINS IN CIRCULATING BLOOD

Classes	Mean increase ± S.E. (mg. per cent)		Difference between treated and untreated	
	Controls*	Cortisone†	t =	P =
S_f 0 to 12	597±60.1‡	343±88.6‡	2.37	0.03
S_f 12 to 20	311±31.9‡	90±28.8	3.56	0.002
S_f 20 to 400	511±172.1‡	1193±222.8‡	2.72	0.015

* Cholesterol-fed (1 gm./day) for 30 days.
† Cholesterol-fed and cortisone, I.M., 3.0 mg./day for 30 days except Sundays.
‡ Significant change between pre-experimental and final values.

The differences between pre-experimental and final (30-day) experimental values of three classes of low-density lipoproteins in the circulating blood of untreated and cortisone-administered, cholesterol-fed rabbits and the effect of cortisone are shown in TABLE 10. In the untreated group lipoproteins of S_f 0 to 12, 12 to 20, and 20 to 400 classes are significantly elevated from their respective pre-experimental levels. The amounts of S_f 0 to 12 and S_f 20 to 400 lipoproteins in the circulating blood of cortisone-administered rabbits also are significantly increased, while the lipoprotein concentration of the S_f 12 to 20 class is not different from the pre-experimental value. A statistical summarization of the data showing the effect of cortisone on the 3 classes of lipoproteins present in circulating blood at the end of 30 days of a high-cholesterol diet is presented in TABLE 10. Cortisone administration resulted in levels of S_f 0 to 12 and 12 to 20 lipoproteins, which are significantly less, while the lipoprotein concentration of S_f 20 to 400 class is significantly greater than that of animals fed a high cholesterol diet alone. The findings agree with those observed[12] in the normal rabbit treated with cortisone and suggest[12] the cause to be a "block" in the conversion of high-S_f classes to those of classes below S_f 80. Moreover, these data suggest development of a discrepancy in the catabolism of low-density lipoproteins at an early period of cortisone administration to rabbits fed a high-cholesterol diet.

TABLE 11

EFFECT OF ADDITION OF PLASMAS OF CHOLESTEROL-FED, UNTREATED, AND CORTISONE-TREATED RABBITS ON LIPOPROTEIN LIPASE ACTIVITY *IN VITRO*

Groups	Normal plasma (pre-exper.) fall OD (percentages)	Decrease in clearing activity* Experimental period		
		14 days	30 days	P =
Controls	76.6±0.6	21.7±2.1	29.5±2.9	0.05
Cortisone†	75.6±0.9	20.4±2.4	42.7±2.3	<0.01
P =	ns	ns	<0.01	

* Mean difference of pre-experimental and experimental plasma clearing. Fall OD (per cent) of individuals of each group.

† Cortisone, I.M., 3.0 mg. each day except Sundays.

The longitudinal changes during the experiment and the effect of cortisone on clearing of a standard fat emulsion *in vitro* are of interest (TABLE 11) in view of their relevancy to the defect in lipoprotein metabolism exhibited by rabbits injected with cortisone. The first column shows the mean fall (in percentages) in optical density in mixtures of postheparin plasma with pre-experimental plasma (normal) after 60 min. incubation at 30° C. The plasmas used in this test were obtained from each rabbit destined to be placed either on the high-cholesterol diet alone or in conjunction with cortisone administration. At 14 and 30 days of these regimes, clearing measurements were repeated, using the same pool of postheparin-active plasma. A small but significant decrease of clearing activity *in vitro* was found in mixtures containing plasma obtained from rabbits of both groups after 14 days on their respective regimes. The tests, repeated 2 weeks later, reveal a further small decrease of clearing activity in mixtures of postheparin plasma and plasma from untreated cholesterol-fed rabbits, but the fall in optical density is considerably reduced when plasma from cortisone-injected rabbits is added to the incubation mixture. The data of TABLE 11 also show that cortisone administration caused a very definite inhibition of postheparin clearing activity *in vitro* as compared to the results in mixtures containing plasma from rabbits after 30 days on the high-cholesterol diet alone. The institution of the cholesterol diet apparently results in a change in the plasma of these animals that causes a constant but small decrease in clearing compared with the normal. Administering cortisone to these animals causes an enhancement of the factor(s) responsible for the inhibition to postheparin-activated clearing of fat *in vitro*, particularly during the second and fourth weeks of the injection schedule. Since lipoprotein lipase activity seems to be normally involved in the catabolism of lipoproteins, the accumulation of the high classes of low-density lipoproteins and the large amount of neutral fat in the plasma of the cortisone-administered rabbits might be explained in part by the greater inhibitory property of the plasma of these animals on the normally delactescing* action of heparin-activated plasma.

* Delactescing is the clearing of milky-appearing plasma; specifically, it is the action of a plasma enzyme causing the clearing of plasma containing a large amount of fat.

Discussion and Summary

There is now convincing evidence that cortisone, even in small doses, causes an inhibition of the propensity of cholesterol-fed rabbits to develop aortic atherosclerosis. Furthermore, this was accomplished without gross changes in the size and lipid content of the liver, as was the case with large amounts of administered cortisone.[5, 21, 22] In general, investigations on the pathogenesis of atherosclerosis indicate an accumulation of lipid in the arterial wall that represents the resultant of factors that promote or hinder the deposit of lipid in the intima. Our studies with cortisone suggest that it exerts an influence on the balance of factors resulting in an interference with the deposition of lipids in the aorta. These investigations indicate a correlation between the inhibitory phenomenon and the coexisting accumulation of neutral fat and high-S_f, low-density lipoproteins in circulating blood, an increased inhibition of post-heparin clearing of fat *in vitro*, and indirect evidence of a "metabolic block" in the conversion of high S_f to classes lower than S_f 20 lipoproteins. Considerations of these results suggests that cortisone induces a discrepancy in the lipid-transporting mechanism, probably due to a defect in the lipolytic processes releasing neutral fat from chylomicrons and low-density lipoproteins. This could lead to inadequate removal of these macromolecules from the blood and alteration of conditions in the blood normally required for cholesterol transport into tissues. The retarded development of atherosclerosis in rabbits administered cortisone and fed a high cholesterol diet may be explained, in part at least, by these changes in the lipid-transporting system.

Only a speculative scheme may be proposed for the potential mechanism by which cortisone inhibits development of atherosclerosis (FIGURE 5). Following the demonstration by Constantinides *et al.*[23] of the production of clearing factor after injection of artificially sulfated polysaccharides, Gillman *et al.*[24] showed that naturally derived polysaccharides acted in a similar way. In other investigations[14, 25, 26] it was shown that heparin, a naturally occurring sulfated polysaccharide, inhibited the development of atherosclerosis in cholesterol-fed rabbits. Although it is not certain, the metachromatically staining cytoplasmic granules of the mast cells of connective tissue are believed to be precursors of heparin. That being the case, mast cell function and factors controlling their metabolism would be intimately concerned in lipid metabolism, particularly the lipid-transporting mechanism. There is considerable evidence[27] to show that endocrines play a role in the function of mesenchymal tissue. Mast cells appear to be particularly sensitive to cortisone, which decreases their number and greatly depletes their cytoplasmic granules.[28, 29] Also, Layton[30] and Boström and Odeblad[31] reported a decrease in sulfate fixation in mucopolysaccharides after treating rats with cortisone. It is interesting that *in vivo* studies[32] of sulfate fixation in the cardiovascular system showed that the highest incorporation of S-35 occurred in the aorta, the walls of arteries, and the heart valves. It is suggested that the inhibitory effect of cortisone on incorporation of inorganic sulfate into polysaccharides may well be the bio-

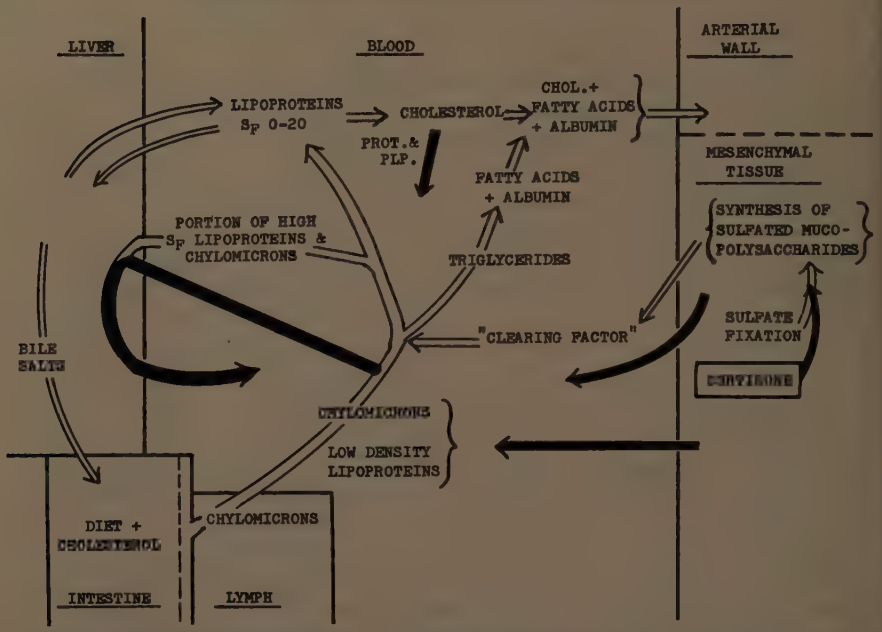

FIGURE 5. Schematic representation of the possible action of cortisone in producing abnormal lipid metabolism. The open arrows indicate the lipid- (cholesterol) transporting mechanism in the untreated cholesterol-fed rabbit. The black arrows show the site of the proposed action of the cortisone on sulfate fixation in mucopolysaccharide synthesis and the consequent change in the normal lipolytic processes resulting in the accumulation of neutral fat and lipoproteins of the high-S_f classes. See text for discussion of the possible relation of this alteration of the normal lipid-transporting mechanism to the inhibition of development of atherosclerosis in cortisone-treated, cholesterol-fed rabbits.

chemical lesion related to the observations of a discrepancy in the lipid-transporting mechanism of cortisone-treated, cholesterol-fed rabbits.

In the scheme put forth the ground substance is proposed as the logical site of action of cortisone. Seifter and Baeder[33] reported the presence of an inhibitor to lipemia clearing in the plasma of cortisone-injected, stressed, and nephrotic rats, while an inhibitor of clearing factor was not found present in the plasma of rats treated with agents that have an opposite effect on the ground substance; namely, hyaluronidase, depolymerized hyaluronic acid, and desoxycorticosterone. If cortisone does, indeed, alter the nature of the metachromatic polysaccharides believed to be the inciting agents for production of clearing factor and a part of its complex, then the normal metabolic reactions of the normally occurring biochemical substance would not be carried out in the blood. One logical test of this hypothesis would be to determine the effect of heparin in the cortisone-treated animal. This has already been done by Constantinides *et al.*,[34] who found that heparin does not inhibit cortisone-induced lipemia in the rabbit and in fact reported that it tended to increase the lipemia. Their results appear more readily explainable in the light of the suggested mode of action of cortisone presented here.

Materials and methods. New Zealand white female rabbits of pedigreed stock were used in these studies. A commercial rabbit food pellet was the regular diet. This was given in portions of 50 gm. per rabbit each morning and afternoon and tap water ad libitum. Cholesterol (Cholesterin) supplementation to the diet was done by spreading 1 gm. of the sterol on 15 gm. of freshly shredded carrots per rabbit. This ration was provided each day except Sunday shortly before the morning portion of food pellets was provided. No attempt was made to dissolve the cholesterol in any solvent. Controls received shredded carrots only. Cortisone acetate (in physiological saline) was administered intramuscularly in the thighs of rabbits, using the left and right legs alternately each day. Different amounts administered were made to volume to inject 0.4 ml. per rabbit; controls received an equal amount of physiological saline each time. The methods and procedures used for the examination of the tissues and determinations of the lipids were those currently in use in this laboratory.[5] Determinations of low-density lipoproteins in blood were done on samples submitted by previous arrangement at the Institute of Medical Physics, Belmont, Calif. *In vitro* clearing measurements were done by the methods of Grossman *et al.*[19] and of Schotz *et al.*[20]

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ADRENAL MEDULLARY HORMONES AND ARTERIOSCLEROSIS*

Y. T. Oester
Stritch School of Medicine, Loyola University, Chicago, Ill.

Introduction

In the field of arteriosclerosis a great many investigators are preoccupied with studies concerning the lipid aspects of this pathology. Many authorities seem to be agreed that these factors are unquestionably very important components of human arteriosclerosis. However, whether lipids are primary factors or even the most important aspects of the disease is still not established. There is considerable evidence in the literature that points to two related facts: first, that nonlipid influences are also of significant importance[1, 2] and, second, that nonlipid involvement of the media of the arterial wall is a regular post-mortem finding in arteriosclerosis.[3, 4]

In a search for nonlipid endogenous factors involved in the arteriosclerotic process, the reports of an arteriosclerosis produced in rabbits by the administration of epinephrine seem pertinent. As early as 1903, Josue[5] reported the production of arteriopathy in rabbits after intravenous injections of epinephrine. This finding has been supported adequately and confirmed by a series of subsequent investigations.[6-8] Several other species of animals have been treated with epinephrine, with the resultant production of arteriosclerosis. A similar arteriopathy in the dog has been reported by Otto,[8] by Enger,[9] and by Waters and de Suto-Nagy.[10] Arteriosclerosis was noted in the monkey in one case reported by Boveri.[11]

In cases of epinephrine-producing tumors (pheochromocytomas) in the human, which result in the production of paroxysmal episodes of high epinephrine blood levels, an associated medial arteriopathy might be expected. A survey of the literature reveals that no specific study has been made relating increased epinephrine blood levels to involvement of the media in arteriosclerosis. However, in at least five different reports atherosclerotic involvement is mentioned in post-mortem studies of individuals in whom pheochromocytomas have been present.[12-16]

Several studies[17-19] have reported the incidence of spontaneous arteriosclerosis in rabbits ranging from very low to as high as 15 per cent. This so-called spontaneous sclerosis resembled that induced by epinephrine. Our own control studies of a regular rabbit population are noted below. Currently, in our own studies at least, the factor of spontaneous arteriosclerosis by gross observation is negligible and does not influence the results in any significant way.

The purpose of the research reported here was to investigate some of the factors that govern or control the production of arteriosclerosis by epinephrine. It is conceivable that endogenous epinephrine in animals as well as in humans might well be one of the important factors leading to production of arteriopathy.

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This is especially pertinent in view of the numerous daily stresses and strains, leading to epinephrine release, to which each of us is subject in a modern high-pressure civilization.

Methods

Adult rabbits of either sex and weighing 2 to 3 kg. were used. Intravenous injections of freshly prepared 1:10,000 commercial epinephrine were administered to each animal daily, 6 days per week, for a total of 15 injections over a period of 17 to 18 days. The dose schedule was as follows: 25 μg./kg. for the first 2 injections; 40 μg./kg. for the third and fourth injections; and 50 μg./kg. for each injection thereafter. Additional drugs were usually given by subcutaneous injection.

Gross autopsy examination was directed primarily to the thoracic and abdominal aorta. The appearance of the intimal surface was carefully noted and graded on the basis of sight and touch as follows: (0) smooth and glistening, no lesions; (1) one to several small "pinhead" patches; (2) many small "pinhead" patches; (3) several confluent patches; and (4) widely distributed confluent patches.

Histological examinations were limited chiefly to routine hematoxylin and eosin stain. No attempt was made to grade the severity of the pathology on a microscopic basis.

Comparison among the various experimental groups was made on the following basis:

$$\text{Total per cent incidence of sclerosis (of any degree)} = \frac{\text{number of sclerotic animals}}{\text{total number of animals}} \quad (1)$$

$$\text{Average degree of sclerosis} = \frac{\text{sum of each degree of all four degrees} \times \text{total number of animals (of that degree)}}{\text{total number of animals in the experiment}} \quad (2)$$

$$\text{Severity factor} = \text{per cent incidence} \times \text{degree of sclerosis} \quad (3)$$

Results

Administration of epinephrine alone produced the results shown in TABLE 1. It is clear that in both of the groups that received epinephrine, a significant

TABLE 1
EPINEPHRINE-INDUCED AORTIC SCLEROSIS IN THE RABBIT
(Fifteen Daily Injections)

Group	Percentages of incidence (No. sclerotic/No. animals)	Degree of sclerosis					Average degree	Severity factor
		0	1	2	3	4		
Epinephrine	5/10 = 50	5	4	1	0	0	0.6	35
Epinephrine	10/20 = 50	10	5	3	2	0	0.9	45
Saline	0/24 = 0	24	0	0	0	0	0.0	0
No injection	1/84 = 1	83	1	0	0	0	0.0	0

Dosage: epinephrine, intravenously, 0.05 mg./kg./day.

FIGURE 1. Aorta of rabbit treated with epinephrine alone; degenerated and calcified area in media. Hematoxylin-eosin stain, ×200.

incidence of arteriosclerosis of approximately 50 per cent resulted. The histological appearance of this arteriopathy, primarily an involvement of the media, is shown in FIGURE 1. In addition, a type of intimal proliferation was seen in some animals and is shown in FIGURE 2. In no case was there any discernible accumulation of lipid in the aortas examined from these experiments.

The histological picture is primarily one of involvement of the media. Initially there is a separation or loosening of the medial elastic and muscle components. Later these elements appear to be fragmented and to be invaded

FIGURE 2. Aorta of rabbit treated with epinephrine; some intimal proliferation present. Hematoxylin-eosin stain, ×200.

ultimately by calcium. Some hyaline-type degeneration is also present. Degeneration and necrosis summarize these changes. A coexisting proliferative appearance in the intima was also present in a number of cases but, in general, this proliferation was minimal to moderate. Foam cell formation was not noted, nor was there any evidence of sudanophilic material in these lesions.

Certain of the pharmacological effects of epinephrine are augmented or potentiated in the presence of thyroxin; this is true for the epinephrine blood

TABLE 2

EPINEPHRINE-THYROXIN-INDUCED AORTIC SCLEROSIS IN THE RABBIT

(Fifteen Daily Injections)

Group*	Percentages of incidence (No. sclerotic/No. animals)	Degree of sclerosis					Average degree	Severity factor
		0	1	2	3	4		
EPIN	5/10 = 50	5	4	1	0	0	0.6	30
EPIN + $THYR_1$	8/10 = 80	2	1	2	3	2	2.2	176
EPIN + $THYR_2$	9/10 = 90	1	1	1	3	4	2.8	252
EPIN + $THYR_3$	4/5 = 80	1	0	1	2	1	2.4	192
$THYR_2$	0/10 = 0	10	0	0	0	0	0.0	0
$THYR_3$	0/4 = 0	4	0	0	0	0	0.0	0

* Epinephrine (EPIN), I.V., 0.05 mg./kg./day. Thyroxin (THYR), S.C.: $THYR_1$, 0.05 mg./kg./day; $THYR_2$, 0.15 mg./kg./day; $THYR_3$, 0.25 mg./kg./day.

pressure effect in the dog. With this in mind, the possibility that thyroxin might augment the epinephrine arteriopathy was considered. The addition of daily subcutaneous injections of thyroxin to the epinephrine regimen of intravenous injections resulted in a markedly augmented production of medial arteriosclerosis. The over-all incidence was raised to 90 per cent in comparison to 50 per cent produced by epinephrine alone; these data are presented in TABLE 2. The gross appearance of the lesions of the aorta was more extensive and more severe than with the administration of epinephrine alone. Histologically (FIGURE 3), except for the increased severity of the lesions, they were like those caused by epinephrine alone.

The substitution of triiodothyronine, which is thought to be the form in which thyroxin produces some of its effects at the cellular level, was used in two groups of animals (TABLE 3). At a dosage level of 0.05 mg./kg., an 80 per cent incidence of arteriopathy was produced. This indicates that this compound could substitute quite satisfactorily for thyroxin in producing augmentation of the epinephrine arteriopathy.

In an endeavor to evaluate the effect of stimulation of the thyroid gland on the production of arteriosclerosis, daily intravenous injections of thyroid-stimulating hormone (TSH) were added to the epinephrine regimen. One group was given 0.05 unit per day, while a second group received 0.10 unit per day; the results of this experiment are shown in TABLE 4. Some toxic effects of TSH were observed at these dose levels, but neither dose augmented epinephrine-induced arteriosclerosis.

Dinitrophenol is an agent that produces an increase in the metabolic rate somewhat similar to that produced by thyroxin. The administration of dinitrophenol in a dose of 40 mg./kg./day failed to augment the epinephrine sclerosis (TABLE 4). The finding that neither thyroid-stimulating hormone nor dinitrophenol augmented the epinephrine sclerosis would indicate that increased metabolic rate per se is not involved in the thyroxin effect.

A series of 13 rabbits was thyroidectomized and then given the epinephrine regimen. In none of these animals was there even minimal evidence of aortic sclerosis. This result, coupled with the finding that even in toxic doses thy-

FIGURE 3. Aorta of rabbit treated with epinephrine-thyroxin; some intimal proliferation and severe medial necrosis. Hematoxylin-eosin stain, ×200.

roxin alone failed to produce sclerosis, would indicate that both hormones are essential to the production of the epinephrine arteriosclerosis as it was studied here.

Suppression of thyroid gland activity is known to result from the administration of propylthiouracil. TABLE 5 presents the protocol of an experiment in which 125 mg. propylthiouracil/kg./day was given orally as a supplement to the epinephrine regimen. Each of the animals in this group was found to have

TABLE 3

EPINEPHRINE-TRIIODOTHYROXIN-INDUCED AORTIC SCLEROSIS IN THE RABBIT

(Fifteen Daily Injections)

Group*	Percentages of incidence (No. sclerotic/No. animals)	Degree of sclerosis					Average degree	Severity factor
		0	1	2	3	4		
EPIN	5/10 = 50	5	4	4	0	0	0.6	30
EPIN + TR_1	3/9 = 33	6	2	1	0	0	0.4	13
EPIN + TR_2	8/10 = 80	2	1	0	4	3	2.5	200

* Epinephrine (EPIN), I.V., 0.05 mg./kg./day; triiodothyroxin (TR_1), S.C., 0.01 mg./kg./day; TR_2, S.C., 0.05 mg./kg./day.

TABLE 4

EPINEPHRINE-INDUCED AORTIC SCLEROSIS WITH DINITROPHENOL AND THYROID-STIMULATING HORMONE IN THE RABBIT

(Fifteen Daily Injections)

Group*	Percentages of incidence (No. sclerotic/No. animals)	Degree of sclerosis					Average degree	Severity factor
		0	1	2	3	4		
EPIN	5/10 = 50	5	4	1	0	0	0.6	30
EPIN + DNP	4/10 = 40	6	2	2	0	0	0.6	24
EPIN + TSH	3/8 = 37	5	2	1	0	0	0.5	18

* Epinephrine (EPIN), I.V., 0.05 mg./kg./day; dinitrophenol (DNP), S.C., 40.0 mg./kg/day; thyroid-stimulating hormone (TSH), I.V., 0.1 unit/kg./day.

TABLE 5

EPINEPHRINE-PROPYLTHIOURACIL-INDUCED AORTIC SCLEROSIS IN THE RABBIT

(Fifteen Daily Injections)

Group*	Percentages of incidence (No. sclerotic/No. animals)	Degree of sclerosis					Average degree	Severity factor
		0	1	2	3	4		
EPIN	5/10 = 50	5	4	1	0	0	0.6	30
EPIN + PTU	10/10 = 100	0	1	8	1	0	2.0	200
PTU	5/9 = 56	4	1	3	1	0	1.1	62

* Epinephrine (EPIN), I.V., 0.05 mg./kg./day; propylthiouracil (PTU), orally, 125 mg./kg./day.

aortic sclerosis: an incidence of 100 per cent. Surprisingly, when propylthiouracil alone was administered to a group of animals, an incidence of 50 per cent was found. The type of pathology that resulted was not essentially different histologically from that produced by epinephrine alone.

It has been extremely difficult to produce cholesterol arteriosclerosis in higher animals such as the dog. Since 1946, several investigators[20-24] have reported the production of arterial sclerosis in the dog by prolonged simultaneous feeding of cholesterol and thiouracil or propylthiouracil. Only one group of investigators[21] in this series of studies reported any control uracil-treated dogs.

TABLE 6

EPINEPHRINE- AND ARTERENOL-INDUCED AORTIC SCLEROSIS IN THE RABBIT
(Fifteen Daily Injections)

Group*	Percentages of incidence (No. sclerotic/No. animals)	Degree of sclerosis					Average degree	Severity factor
		0	1	2	3	4		
EPIN (commercial)	10/20 = 50	10	5	3	2	0	0.9	43
EPIN (100 per cent)	10/21 = 48	11	7	3	0	0	0.6	29
Arterenol (100 per cent)	4/23 = 17	19	1	0	2	1	0.5	6
Arterenol + thyroxin	17/22 = 77	5	2	6	7	2	2.0	154
EPIN + thyroxin	17/20 = 85	3	1	6	7	3	2.3	196

* Epinephrine (EPIN) and arterenol, I.V., 0.05 mg./kg./day; thyroxin, S.C., 0.15 mg./kg./day.

Four such dogs were reported as negative for arteriosclerosis after prolonged thiouracil treatment. All of the investigators in these experiments were of the opinion that the uracils mediated their atherogenic effect by way of the hypothyroid state and the resulting hypercholesterolemia. From the findings of the present study, wherein rabbits developed arteriosclerosis while being fed propylthiouracil alone, it is justifiable to raise the question as to whether the arteriosclerosis in the experiments on dogs may not have been due to a direct effect of the antithyroid drug itself rather than to the indirect effect of hypothyroidism.

The commercial epinephrine used in the early experiments reported here was later shown to contain 10 to 15 per cent of arterenol. To investigate the effect of this preparation in contrast to pure epinephrine and pure arterenol, groups of animals were treated with these agents. The experimental data are presented in TABLE 6. It is apparent from these data that both commercial epinephrine containing some arterenol and pure epinephrine produce arteriosclerosis, the incidence being approximately 50 per cent. The pure epinephrine, therefore, is as effective as the impure in producing this effect.

Pure arterenol alone in the same doses as those used for epinephrine was less effective in producing sclerosis than was pure epinephrine. The incidence of sclerosis production for arterenol was 17 per cent; for epinephrine the incidence was 48 per cent. When thyroxin was added to each of these regimens, the incidence percentages for arterenol plus thyroxin and for epinephrine plus thyroxin were 77 and 85, respectively. There thus appears to be a considerable difference in sclerogenic activity between the two pure sympathomimetic amines alone but, with concomitant thyroxin administration, the sclerosis produced by each is markedly augmented. This augmentation was such that, even though not identical, there was no significant difference between the two groups. Histologically, as shown in FIGURE 3, there was no difference between the arteriosclerosis produced by these two agents.

The effect of the epinephrine-thyroxin regimen upon the blood cholesterol and phospholipid levels was determined. TABLE 7 presents the protocol from this experiment. There was no significant change in cholesterol or phos-

TABLE 7

CHOLESTEROL AND PHOSPHOLIPID PLASMA VALUES IN EPINEPHRINE-THYROXIN-INDUCED AORTIC SCLEROSIS

Data from 41 Rabbits, in mg./100 ml.

Determination		Time in weeks			
		0	1	2	3
Cholesterol	Av.:	65.9	68.3	63.5	67.3
	Range:	(59.4–77.2)	(60.1–76.4)	(55.1–68.7)	(55.1–80.6)
Phospholipids	Av.:	100.7	121.0	97.3	99.0
	Range:	(74.8–113.2)	(89.4–169.8)	(77.2–127.5)	(71.6–125.1)
Cholesterol/-phospho-lipid ratio	Av.:	0.65	0.56	0.65	0.68
	Range:	(0.56–0.84)	(0.41–0.67)	(0.52–0.79)	(0.59–0.77)

TABLE 8

EFFECT OF TREATMENT WITH VARIOUS AGENTS ON EPINEPHRINE-THYROXIN-INDUCED AORTIC SCLEROSIS IN THE RABBIT

(Fifteen Daily Injections)

Group*	Percentages of incidence (No. sclerotic/No. animals)	Degree of sclerosis					Average degree	Severity factor
		0	1	2	3	4		
EPIN + THYR	43/48 = 89	5	4	10	13	16	2.6	233
Heparin	9/17 = 53	8	0	2	3	4	2.0	190
α-Tocopherol	6/8 = 75	2	0	1	2	3	2.5	187
Progesterone	9/11 = 82	2	1	2	2	4	2.5	205
AMP	10/13 = 77	3	0	3	2	5	2.5	195
ATP	3/14 = 21	11	0	1	2	0	0.6	12

* Epinephrine (EPIN), I.V., 0.05 mg./kg./day; thyroxin (THYR), S.C., 0.15 mg./kg./day; heparin, I.V., 5000 units total dose per day; α-tocopherol, S.C., 600 gm./kg./day; progesterone, S.C., 75 mg./kg./day; adenosine monophosphate (AMP), S.C., 40 mg./kg./day; adenosine triphosphate (ATP), S.C., 200 mg./kg./day.

pholipid blood levels during the course of the development of the arteriosclerosis produced by the epinephrine-thyroxin treatment.

A long-range goal of the experimental work reported here was the investigation of agents that might prevent the epinephrine-thyroxin sclerosis or, once it had developed, produce regression of the sclerosis. To this end a number of the agents widely suggested for use in the clinical treatment of arteriosclerosis were given to rabbits concomitantly with the regular epinephrine-thyroxin regimen; the results of this study are shown in TABLE 8. Adenosine triphosphate (ATP) by subcutaneous injection was the only agent that produced a significant diminution in the incidence of arteriosclerosis.

Since ATP was the only substance producing a preventive effect, it was decided to use it in an endeavor to cause regression of an established epinephrine-thyroxin sclerosis. For this purpose a group of 60 rabbits was given the routine epinephrine plus thyroxin on a 15-day regimen that would produce an incidence of sclerosis of approximately 90 per cent. Thirty of these arterio-

sclerotic rabbits were then given a daily subcutaneous dose of ATP of 200 mg./kg. This was continued for a period of 6 weeks. At the end of this time the incidence of sclerosis for each group was 90 per cent for the controls and 87 per cent for the ATP-treated animals. This indicates that the prolonged ATP treatment had no effect on regression of the epinephrine-thyroxin-induced sclerotic lesions.

Discussion and Summary

The arteriopathy produced in rabbits by epinephrine administration does not seem to be related directly to the blood pressure effect of epinephrine. This pressor action is very evanescent in nature. In addition, arterenol, which is considered to be a much more effective pressor agent, was less potent in its sclerogenic effect. The pathology also does not seem to be directly related to an effect on basal metabolism, since dinitrophenol did not augment epinephrine sclerosis; however, thyroxin is essential for production of the arteriopathy. A reasonable assumption is that the site of action of the epinephrine-thyroxin effect is at a metabolic level and not directly related to oxygen consumption. This is stated in the light of the absence of effect with dinitrophenol and a consideration of the action of ATP in inhibiting the effect.

The histological character of the arteriosclerosis produced by epinephrine plus thyroxin resembles closely the so-called Mönckeberg's sclerosis or medial arterionecrosis. The picture is that of a sclerosis of the media, but with sufficient intimal involvement to suggest that this intimal finding is more than an ancillary one. It is striking to note the similarity between the arteriopathy described here in the aorta and that described by Taylor *et al.*[25] in arteries subjected to physical injury such as freezing. These similarities, resulting from two such dissimilar methods of induction, lend support to the idea that the arterial wall reacts more or less uniformly to injury or trauma, regardless of the nature of the trauma.

The arteriosclerosis described here can be produced as a result of the use of arterenol as well as epinephrine. It can also be augmented by triiodothyronine as well as by thyroxin.

The failure of epinephrine alone to produce the gross arteriopathy when the thyroid gland was removed, and the failure of large doses of thyroxin alone to produce the arteriopathy indicate that both of these types of substances are essential for the production of so-called epinephrine arteriopathy.

Propylthiouracil, when given alone, produced a significant incidence of epinephrinelike arteriopathy. This finding raises perplexing questions in relation to experiments in which uracils and lipids were used to produce an atherosclerosis in the dog, a species ordinarily resistant to such pathology.

Failure to detect any significant change in the cholesterol or phospholipid blood levels during the course of the development of epinephrine-thyroxin arteriopathy would suggest that a mechanism other than hypercholesterolemia is involved in the production of the pathology.

Adenosine triphosphate, the only agent with any significant preventive action in regard to epinephrine-thyroxin sclerosis, did not produce any effect

when used in an endeavor to cause regression of an established epinephrine-thyroxin sclerosis. There is no indication by what mechanism the beneficial preventive effects of adenosine triphosphate were obtained.

The implications of the findings that have been described, relating to experimental arteriopathy in rabbits in relation to the genesis of human atherosclerosis are more or less obvious. These studies indicate that comparatively large amounts of the endogenous hormones epinephrine and arterenol and of thyroxin and triiodothyronine, may consistently produce an arteriosclerosis of the media. A certain degree of intimal proliferation may follow. The growing evidence that a nonlipid involvement of the media is present in association with the intimal and subintimal plaque formation in human atherosclerosis and the finding of frequent medial involvement as a preatheromatous lesion are presumptive indications that some factor not directly related to lipids is acting. In the experiments on animals reported here it has been demonstrated that an agent or combination of agents already present in the rabbit will produce such a nonlipid arteriosclerosis when used in the dosages indicated. Whether appropriate amounts of these physiological substances are endogenously produced is not established by this report. Furthermore, these agents are also present in man.

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DISCUSSION OF PART II

ROBERT W. WISSLER (*Department of Pathology, The University of Chicago, Chicago, Ill.*): I shall begin with two general observations. First, I support the broad perspective of the metabolic and biochemical presentations comprising the first section of this monograph. For a long time students of arteriosclerosis have spoken about cholesterol in the blood while seeking and staining neutral fat in the arteries. It is a very good sign, it seems to me, that other lipid substances in blood and tissue are now receiving increasing attention in relation to atherosclerosis.

Second, as a pathologist, I must emphasize the importance of distinguishing between atherosclerosis on the one hand and medial necrosis (calcification and other such factors) on the other. While necrotizing medial disease of arteries certainly deserves more study, it probably has little relationship to the morbidity and mortality resulting from arteriopathies in man. Insofar as I can see, the major arterial lesions producing pathological effects are the fatty ones. If little or no fatty material is present in the artery, then fibrosis, calcification, and myxomatous changes probably are of relatively little pathological consequence, whether they are present in the media or in the thickened intima.

The presentations on pituitary and adrenal hormones and their relationship to arteriosclerosis represent a potent prescription of experimental pathology formulated and administered by energetic and productive investigators. To facilitate the absorption and retention of this prescription, I propose to formulate a few questions and observations in order to improve perspective and increase understanding in this difficult field.

The accompanying outline (TABLE 1) indicates why we think of atherosclerosis as a multifaceted disease with a number of pathogenetic processes. It is evident that a substantial part of the current knowledge of atherosclerosis, a great deal of it derived from clinical-pathological correlation, can be organized under three headings: (1) dietary factors, (2) general metabolic factors (not necessarily diet-dependent and probably exclusive of arterial metabolism), and (3) local arterial factors. Perhaps we should add, as a fourth heading, factors influencing the intravascular clotting mechanism, including the plasmin system, but thus far there is no wealth of clinical-pathological correlative data to support this.

Endocrine factors, in particular those discussed in this section, probably exert their main effects via the second and third headings, and they may also influence the clotting mechanism.

As FIGURE 1 indicates, these mechanisms by which atherosclerosis develops are probably independent, exerting their influences singly or together and, frequently, in an additive or synergistic manner. For example, a diet that will prevent or reverse the disease in a patient with no metabolic defect (FIGURE 1, first bar) will not necessarily prevent or reverse atherosclerosis in a patient with metabolic hyperlipemia (FIGURE 1, fourth bar).

Our own recent experimental effort has been largely confined to a study of interrelationships among a number of factors in relation to the production of fatty deposits in the arteries of the rat.

TABLE 1

CORRELATION OF ATHEROSCLEROSIS WITH VARIOUS FACTORS

(1) Association of atherosclerosis with diet:
  (a) correlation with obesity and/or dietary lipid intake;
  (b) variation in severity with the general state of nutrition of the population.
(2) Association of atherosclerosis with the state of lipid metabolism:
  (a) increased severity in diabetes, nephrosis, and xanthomatosis;
  (b) natural protection of the female.
(3) Association of atherosclerosis with vascular connective tissue damage:
  (a) increased severity in areas of luetic aortitis;
  (b) increased severity above aortic coarctations;
  (c) increased pulmonary artery sclerosis in mitral stenosis.

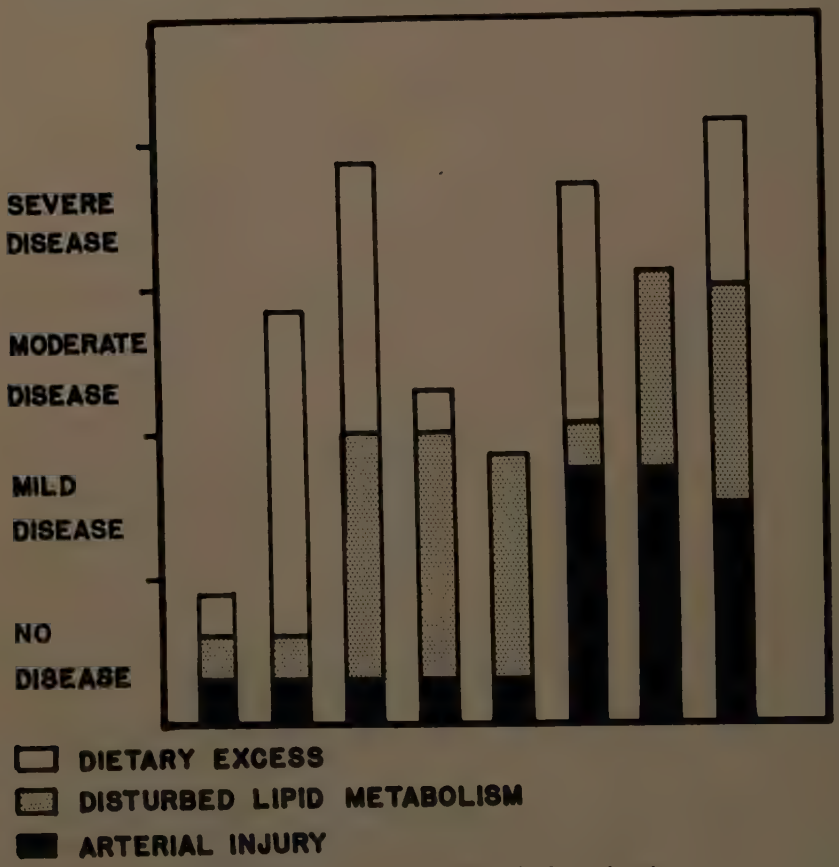

FIGURE 1. Patterns in production of atherosclerosis.

These studies have indicated that the nephrotic syndrome produced by nephrotoxic serum in the rat will result in nearly a 100 per cent incidence of widespread coronary artery intimal lipid deposition,[1] especially when desoxycorticosterone acetate (DCA) and NaCl are administered daily after the

glomerulus has been injured.[2] We believe that at least some of the evidence indicates that DCA and NaCl are producing localized arterial injury that interacts with the lipid metabolic derangement resulting from the nephrotoxic serum. We have found that an entirely different dietary regimen is necessary to prevent or reverse these arterial lesions as compared to the diet that prevents or reverses the atheromatous lesions resulting from the prolonged feeding of a high-fat, high-choline, cholesterol-containing ration. Specifically, the results indicate that a low-protein, low-fat ration will prevent or reverse the arterial lipid deposition in the rats treated with nephrotoxic serum, DCA, and NaCl, while a low-fat, high-protein diet will not produce either effect.[3] On the other hand, the latter type of ration will prevent arterial lipid deposition in animals subjected to dietary manipulation only. We believe that these results offer excellent experimental evidence that interrelationships among various pathogenetic processes are very important in any consideration of what causes or reverses atherosclerosis. It follows that patients with definite defects in lipid metabolism or strong evidence of arterial connective tissue injury should be considered separately in therapeutic studies in which the effects of diet or other measures are evaluated. Failure to evaluate therapy separately in each of these various types of patients may lead to conclusions that the measures being tested are not effective. With these considerations in mind and in relation to the stimulating presentations in this section of this publication, I raise the following four questions.

(1) I expect that Salgado and Wilgram will agree that we are just at the beginning of the many possible studies that can be done to relate cardiovascular disease in general and atherosclerosis in particular to specific hormonal and dietary imbalances. The rat is a very convenient animal for this work and, as we have just seen, these two investigators have produced remarkable changes in the heart and the arteries. The problem of how to equate experimental rat disease with human disease is a difficult one. Certainly, DCA and aldosterone seem to have different roles in the rat than in man. Growth hormone must be similarly evaluated. Extension of these studies should help a great deal in understanding arterial and cardiac metabolism but, like much basic biological research, its application to human disease may come by a circuitous route.

(2) Dury's challenging explanation of the cortisone effects that he has observed should be tested in a less susceptible species than the rabbit. I suggest the monkey, and I also suggest that the plasmin (fibrinolysin) mechanism should be studied in relation to his findings.

(3) Adrenalin needs further study in relation to blood lipids and fatty lesions. Although Oester's rabbit lesions are practically confined to the media, there is still a good possibility, I feel, that the recent results reported by Kaplan *et al.*[4] indicate that adrenalin may have a role in atherosclerosis also.

(4) We must know much more about how growth hormone, ACTH, cortisone, DCA, and adrenalin affect lipid metabolism and how they alter the integrity of connective tissue; I am sure that we can count on additional expert help from the biochemists in this endeavor. In particular, we need to develop methods that will help us to recognize the patients who have significant metabolic effects caused by imbalances of these potent substances.

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Part III. Gonadal, Thyroid, and Pancreatic Hormones: Lipids and Arteriosclerosis

CAUSATIVE RELATIONSHIPS OF PARATHYROID HORMONE TO RENOGENIC AND RENIPRIVAL CARDIOVASCULAR DISEASE*

David Lehr

The New York Medical College, Flower and Fifth Avenue Hospitals, New York, N. Y.

It is generally recognized that hyperactivity of the parathyroid glands leads to demineralization of the skeleton and may result in the deposition of calcium salts in the renal tubules, in the myocardium, in the arterial tree, in the stomach wall, and in other soft-tissue sites.[1] This laying down of lime salts is widely believed to take place in healthy tissue as a consequence of the simple physical phenomenon of precipitation from a supersaturated solution. It is distinguished by the term "true metastatic calcification" from "dystrophic calcification," which designates the encrustation of necrotic tissue debris with calcium salts.[2, 3]

Hyperparathyroidism has also been implicated as a cause of hypertension,[4] and a vasoactive factor has been described in parathyroid hormone.[1, 5] However, since the kidneys are invariably damaged in this situation, whether as a result of excess parathyroid hormone (primary hyperparathyroidism) or as the original stimulus for its overproduction (secondary hyperparathyroidism), it is difficult to distinguish the contribution of renal impairment to the hypertensive mechanism from a possible participation of the parathyroid hormone.

In the course of studies on the prevention of renal sulfonamide injury in albino rats, my interest in the parathyroid gland was aroused by the combination of severe nephrocalcinosis, massive calcification of the aorta and its ramifications, and distinct hypertrophy of the parathyroid gland.[6, 7] In exploring this lead with improved procedures for the production of experimental arteriosclerosis by renal injury,[8-10] it was found in our laboratory that extensive disseminated necrosis in the myocardium, in the arterial media, and in the muscularis of the gut invariably preceded demonstrable deposition of calcium.

Subsequently it was shown that the development of muscular necrosis and its sequelae could be readily prevented by prior thyroparathyroidectomy.[11] Studies directed toward elucidation of the mechanism underlying the striking protective effect of thyroparathyroidectomy (TPx) led to the conclusion that the cardiovascular and smooth muscle injury was dependent upon the presence of excess parathyroid hormone.[12]

On further analysis it was demonstrated that hyperfunction of the parathyroid gland resulted from the breakdown of renal function rather than from a toxic substance released by the damaged kidney; it could therefore be induced by bilateral nephrectomy as readily as by severe renal injury. It was also established that, under both these conditions, adrenal cortical hormones were of importance in triggering or permitting the release of excess parathyroid hor-

* The work reported in this paper was supported by Grants H 890 (C4 to C8) from the National Heart Institute, Public Health Service, Bethesda, Md.

mone.[12-14] The present paper, encompassing the work of many years, is concerned with a description of experimental studies that led to recognition of the role of the parathyroid and adrenal cortex in disseminated muscular necrosis. In view of the fact that impairment of kidney function is known to induce hyperactivity of the parathyroid glands in many species other than the rat, including man, it was felt that recognition of the interplay between adrenal cortex and parathyroid gland in the production of cardiovascular and smooth muscle necrosis and calcification might be of considerable importance beyond the realm of the disease entity designated as hyperparathyroidism.

Materials and Methods

Adult male and female albino rats of the Wistar and Sprague-Dawley strains, weighing between 250 and 350 gm., were used. The animals were maintained on Purina Lab Chow and tap water ad libitum and were housed in individual metabolism cages throughout the experimental period.

Standard renal injury was produced by a method outlined in detail elsewhere.[8] In brief, the procedure entails the intraperitoneal injection of a single excessive dose of sodium acetylsulfathiazole (0.5 gm./kg.). Renal excretion of poorly soluble acetylsulfathiazole causes temporary obstruction of the urinary flow (renal blockage) due to massive deposition of crystals in the tubules of the kidneys. The resulting severe injury to the tubular epithelium in turn initiates a series of characteristic organic and functional changes in the kidney frequently referred to under the term "obstructive nephropathy."

Bilateral nephrectomy was carried out from a midline dorsal approach. No effort was made to increase the reniprival life span by special diets or peritoneal dialysis.

The following endocrine organs were removed: both adrenals, the parathyroids, or the thyroid and the parathyroids. Adrenalectomized rats were maintained on saline up to the time of specific hormonal substitution. Thyroparathyroidectomy was performed with the aid of a binocular loop. No serious postoperative complications were encountered. Four weeks were allowed to elapse between the operation and use of these animals. Parathyroidectomy consisted in thorough excision of all parathyroid tissue visible under the lens, together with small bits of adherent thyroid tissue. In more recent studies, parathyroidectomy was carried out under the dissecting microscope by electrocoagulation, using a fine needle electrode of the Bovie Electrosurgical Unit. This eliminated the complication of afterbleeding, which is encountered in surgical excision of the parathyroid gland, while preserving the integrity of adequate parts of the thyroid gland. Recovery after electrocoagulation was faster than after surgical extirpation; such animals were often used within 1 to 2 days following the procedure. All operative procedures were carried out under ether anesthesia. Finally, for "chemical thyroidectomy," rats received 0.01 per cent propylthiouracil in the drinking water for a minimum of 4 weeks preceding their use in experiments.

Various situations of single or multiple hormone deficiency or excess were created by combining the removal of endocrine glands with elective and graded substitution of specific endocrine factors. All hormone preparations were administered by the subcutaneous route. In rats with renal injury, cortisone* alone or in conjunction with desoxycorticosterone acetate (DCA)* was injected in daily dosages of 3 mg. each, starting 3 days prior to initiation of the renal damage and continuing for a minimum of 3 days thereafter. Similarly, parathyroid extract† was injected for partial to full substitution or poisoning in daily dosages ranging from 10 to 200 I.U., starting 5 days prior to standard renal injury (kidney block) and continuing for at least 5 more days.

In the nephrectomy studies cortisone and DCA were injected individually as well as in combination in daily dosages of either 3 or 10 mg., starting 2 days prior to nephrectomy and continuing daily for the remaining life span of the animal. Parathyroid was administered either throughout the reniprival life span or only on the first and second days postnephrectomy in amounts of 100 and 200 I.U. daily.

In thyroparathyroidectomized rats the missing thyroid was replaced by daily substitution with 2.5 or 5 mg. of dried thyroid injected as a suspension of the powder.

The systolic blood pressure was recorded with the help of a photoelectric tensometer that measures the volume change in the hind leg of the unanesthetized animal.[15] Serial measurements at predetermined intervals were done in animals with renal injury. The average of 3 to 6 satisfactory successive readings obtained by the same experimenter was considered to constitute the systolic blood pressure. Because of the strain of immobilization entailed by this procedure, it was not possible to expose nephrectomized rats routinely to repeated blood pressure measurements, since in such animals the survival time was a crucial point, and a definite shortening of the reniprival life span was observed when repeated blood pressure readings were taken; hence the determination of the blood pressure in nephrectomized rats was carried out in special duplicate studies.

Serial microdeterminations of calcium,[16, 17] inorganic phosphate,[18] cholesterol,[19] and total carbon dioxide content,[20] as well as terminal estimation of the N.P.N. level,[16] were performed in the blood plasma of each rat. Tail blood served for serial determinations, whereas neck blood or heart blood obtained at sacrifice was used for terminal assays.

Complete post-mortem examinations were performed on every animal. The wet organ weight of heart, thymus gland, adrenals, and kidneys was determined to the nearest milligram on a torsion balance immediately upon removal. All important organs were fixed in formalin and sectioned for microscopic study, using routine and special stains. In recent studies the heart, arterial tree, gastrointestinal tract, and kidneys were examined at autopsy under the dissecting microscope.

* Supplied by Edward Henderson, Schering Corporation, Bloomfield, N.J.
† Obtained from Glenn W. Irwin, Eli Lilly and Company, Indianapolis, Ind.

Results

Renogenic Cardiovascular Disease

Signs Following Renal Obstruction

The typical course of events after standard renal injury is briefly as follows. For the first 24 hours after renal obstruction the rats are oliguric or anuric. Thereafter, usually passing through a stage of microscopic or gross hematuria, all animals develop increasingly excessive polyuria, which remains as a permanent sign of irreversible tubular injury.

For several days the animals appear severely ill, eat very little, and lose weight. The renal injury is reflected early in the blood, where one finds transient elevation of the plasma cholesterol, marked depression of the CO_2-combining power, a rise in N.P.N. and inorganic phosphate levels, and subnormal calcium concentrations. By about the tenth to fifteenth day of the disease the cholesterol and calcium levels may be found to have reverted to normal, whereas the inorganic phosphate levels are still 2 to 3 times above the normal range. At this time N.P.N. levels have usually risen to between 80 and 260 mg. per cent.

The blood pressure rises to hypertensive levels, starting, as a rule, at about the third to fifth day after the renal injury and climbing to average systolic pressure values of 200 mm. Hg at about 2 weeks. Thereafter there is a gradual return to normotensive levels, most animals reaching their original normal blood pressure about 3 to 4 weeks after the injury.

Mean systolic blood pressure levels and daily urine volumes of a group of 15 male rats are illustrated in FIGURE 1. In general, individual rats showed no substantial deviation from the indicated mean.

Pathologic-Anatomical Changes

The nature and incidence of alterations in various organs following renal injury have been described elsewhere.[6, 8-11, 21, 22] Marked hypertrophy of the parathyroids and of the adrenal cortex and atrophy of the thymus gland were invariable features of the experimental disease. The most important damage consisted in extensive necrosis and calcification in the arterial tree, the myocardium, and the muscularis of the gastrointestinal tract. Since the muscular injury at these 3 predilection sites served as the criterion for the evaluation of endocrine and other influences upon their production, the appearance of these lesions and the time relationships in the various stages of their evolvement will be outlined briefly.

Arterial tree. In the aorta and its major and minor branches the primary injury consisted in degeneration of the smooth muscle fibers in subintimal or, more often, in the deeper strata of the aortic media, followed by widespread necrosis, hyalinization, and calcification, which in the majority of animals resulted in the formation of a tubular layer of advanced structural changes from the origin of the aorta at the heart down through its thoracic and abdominal course and extending into the larger and smaller branches of the arterial tree (FIGURE 2). All major branches of the aorta usually were affected severely, but

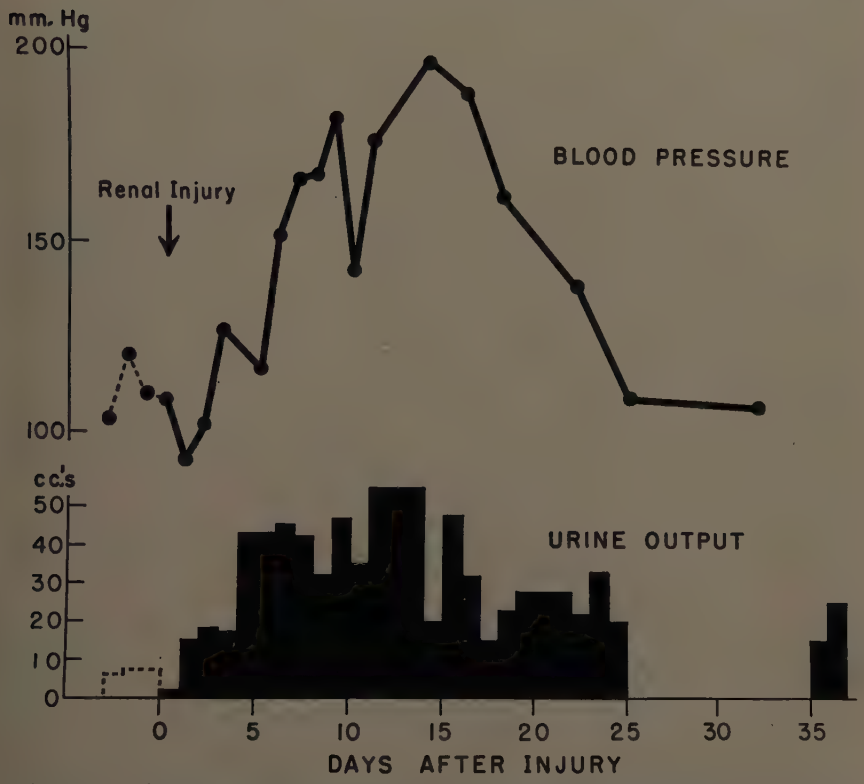

FIGURE 1. Mean systolic blood pressure and mean daily urine volume (per animal) of 15 male albino rats before and after renal injury. Each point of the blood pressure curve represents the average of 4 to 6 consecutive readings.[10] Reproduced by permission of *The Journal of the Mount Sinai Hospital, New York.*

damage of equal magnitude was encountered in the coronary system and in the medium-sized and small arteries of the mesentery (FIGURE 3), the gastrointestinal tract, the kidney, and skeletal muscle.

In striking contrast, the arteries of the smaller circuit were affected far less severely and less often than the vessel of the larger circulation, suggesting a protective influence of lower intravascular tension.

The lesions in the arterial tree were clearly recognizable within 4 to 5 days following renal injury. They started with hydropic degeneration of muscle fibers, disappearance of myofibril structure, pyknosis, and loss of nuclei, fragmentation, and disintegration of necrotic muscle fibers and finally calcification. At the areas of calcification there was accumulation of metachromatic amorphous material that gave a strongly positive periodic acid-Schiff reaction. The pressure caused by the swollen, basophilic, necrotic muscular debris appeared to initiate destruction of elastic lamellae, which also turned strongly basophilic, lost their characteristic parallel pattern, became spread apart, wrinkled, and fragmented (FIGURE 4), and finally encrusted with

FIGURE 2. Medionecrosis and "bamboo-stick" calcification of the stem and large branches of the arterial tree in the rat. Note the rigidity of the aortic arch and the broken-off stump of the celiac artery (pointing toward the cross section of the right kidney). Also visible are the greatly enlarged adrenal glands and kidneys. The rough granular surface of the kidney (right) and the pattern of whitish streaks in the cross section (left) exemplify the typical picture of severe nephrocalcinosis.[22] Reproduced by permission of *Wiener Klinische Wochenschrift.*

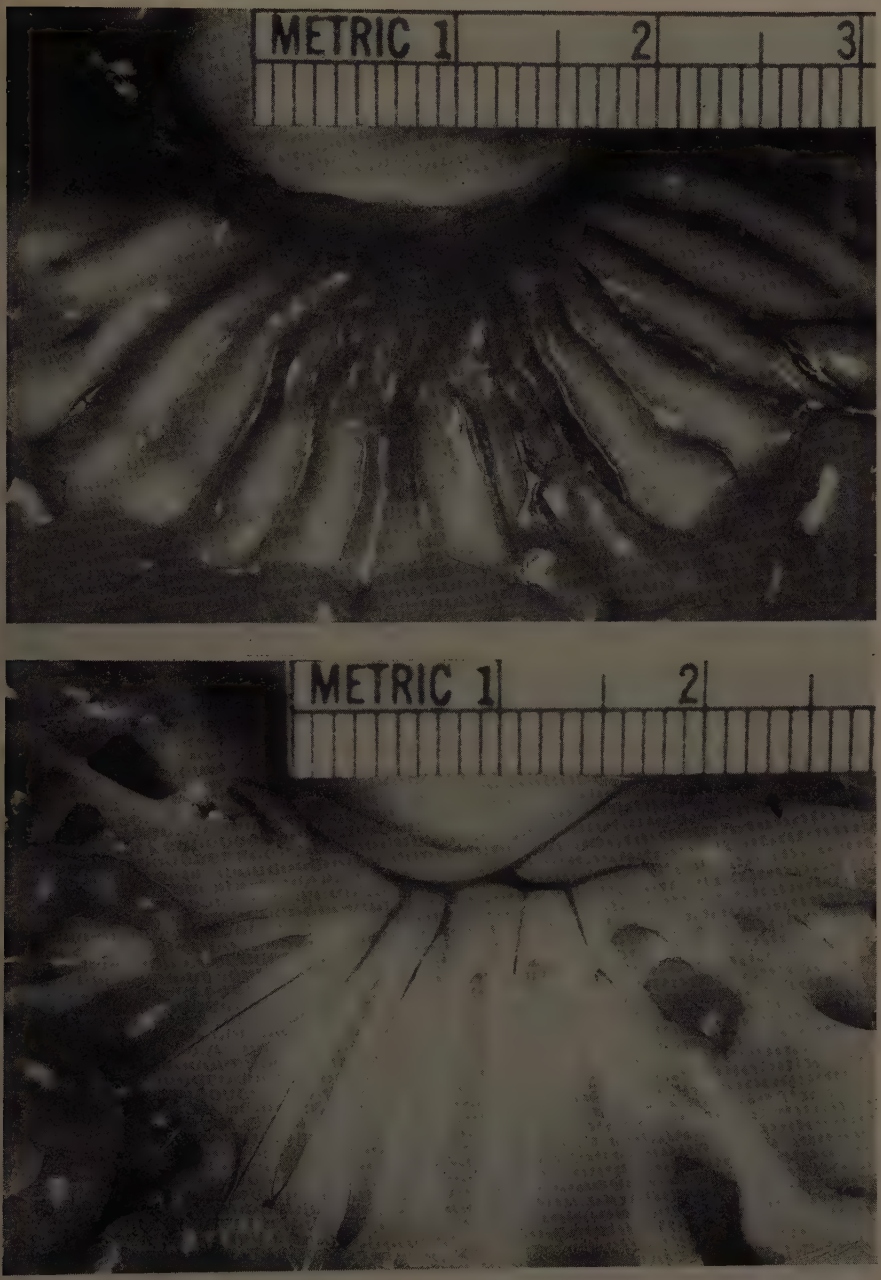

FIGURE 3. Blood vessels in mesentery of the rat. *Top*, note greatly enlarged, thickened, rigid, and curled blood vessels on the sixteenth day following standard renal injury; *bottom*, compare with fine, hardly visible mesenteric arterial branches of control littermate which appear like hairline pencil marks next to the more distinct mesenteric veins.[11] Reproduced by permission from *Endocrinology*.

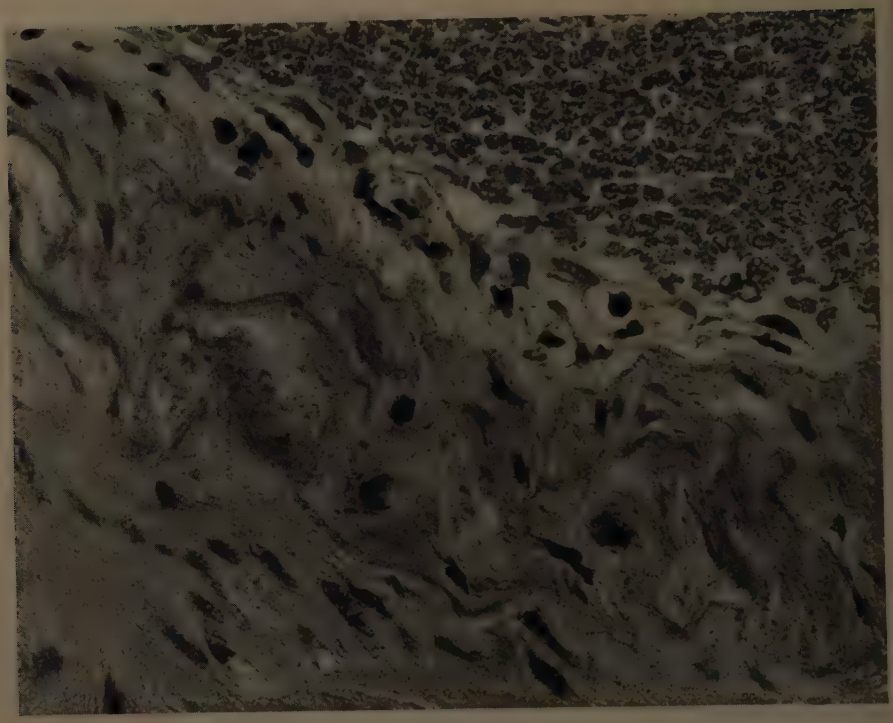

FIGURE 4. Aorta of rat 4 days following renal injury. Intimal proliferation over subintimal necrosis of the media. Note the accumulation of metachromatic amorphous material in the media, the degeneration of muscle fibers, with pyknosis and loss of nuclei, and the destruction of elastic lamellae, which are swollen, spread apart, wrinkled, and fragmented, and are beginning to turn basophilic. The intima over this entire area is edematous and shows doubling and trebling of the normally unicellular layer. Hematoxylin-eosin stain, high magnification.

calcium salts (FIGURES 5 and 6). The consequent weakening of the arterial wall and, particularly, the loss of its elasticity were apparently responsible for extensive diffuse or saccular aneurysmic dilatations (FIGURE 7). These aneurysms were believed to result from the physiological stresses of intravascular tension and pulsatile flow upon a damaged vascular wall. They would thus be enhanced by hypertension and increased pulse pressure. The thinned-out aneurysmic areas showed hyalinization, with complete loss of the normal structure (FIGURE 8). These severe changes in the media together with endothelial and subendothelial proliferation, especially over areas of subintimal necrosis, gave the intimal surface a "tree-bark" appearance (FIGURE 9). Despite the severity of this degenerative process in the aortic media there was often little or no inflammatory response.

At times, about a week after renal injury, the adventitia and the periaortic fat showed diffuse or focal infiltrations with mostly mononuclear cells. These inflammatory reactions occurred more frequently in the medium-sized and

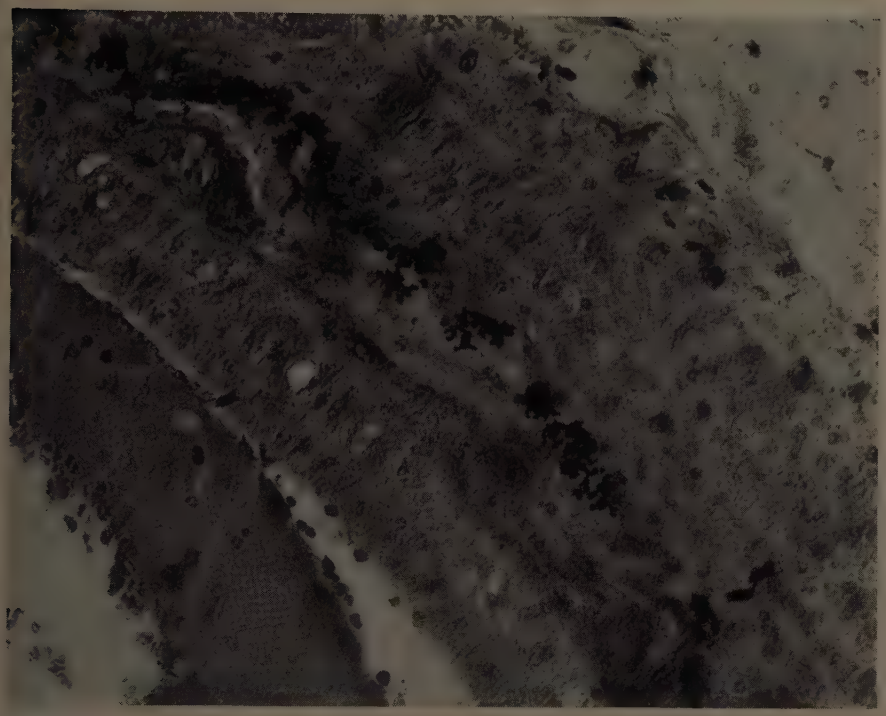

FIGURE 5. Aorta of the rat 5 days after renal injury. Necrosis of the deeper strata of the aortic media (more advanced than that shown in FIGURE 4), with granular calcification of the necrotic debris. Van Kossa calcium stain, high magnification.

small arteries. Muscular necrosis in the media was also more conspicuous (FIGURE 10), and healing resulted in extensive vascular and occasionally perivascular fibrosis.

Within 7 to 10 days calcium imbibition of necrotic areas became prominent. In short segments of the aortic media the swollen debris formed bulging rings of necrosis encompassing the entire circumference (FIGURE 2). This was followed by deposition of connective-tissue ground substance, proliferation of fibroblasts, and the formation of collagen scars. After several weeks these scars became intensely calcified and, at times, they manifested cartilaginous and bony metaplasia (FIGURE 11).

The damage in the arterial tree was often clearly visible to the naked eye even in vessels of the size of the coronary artery. The aorta and its main branches were converted into inelastic thickened friable tubes. Occasionally hyalinization, especially of the dilated aortic arch, was so advanced that this structure assumed the brittleness and transparency of glass. In general, the injury was more pronounced in areas of intensified mechanical stress.

Diffuse injury produced the picture of a "pipestem" aorta, whereas sectional damage, resulting in the formation of calcified bulging rings, gave the aorta a

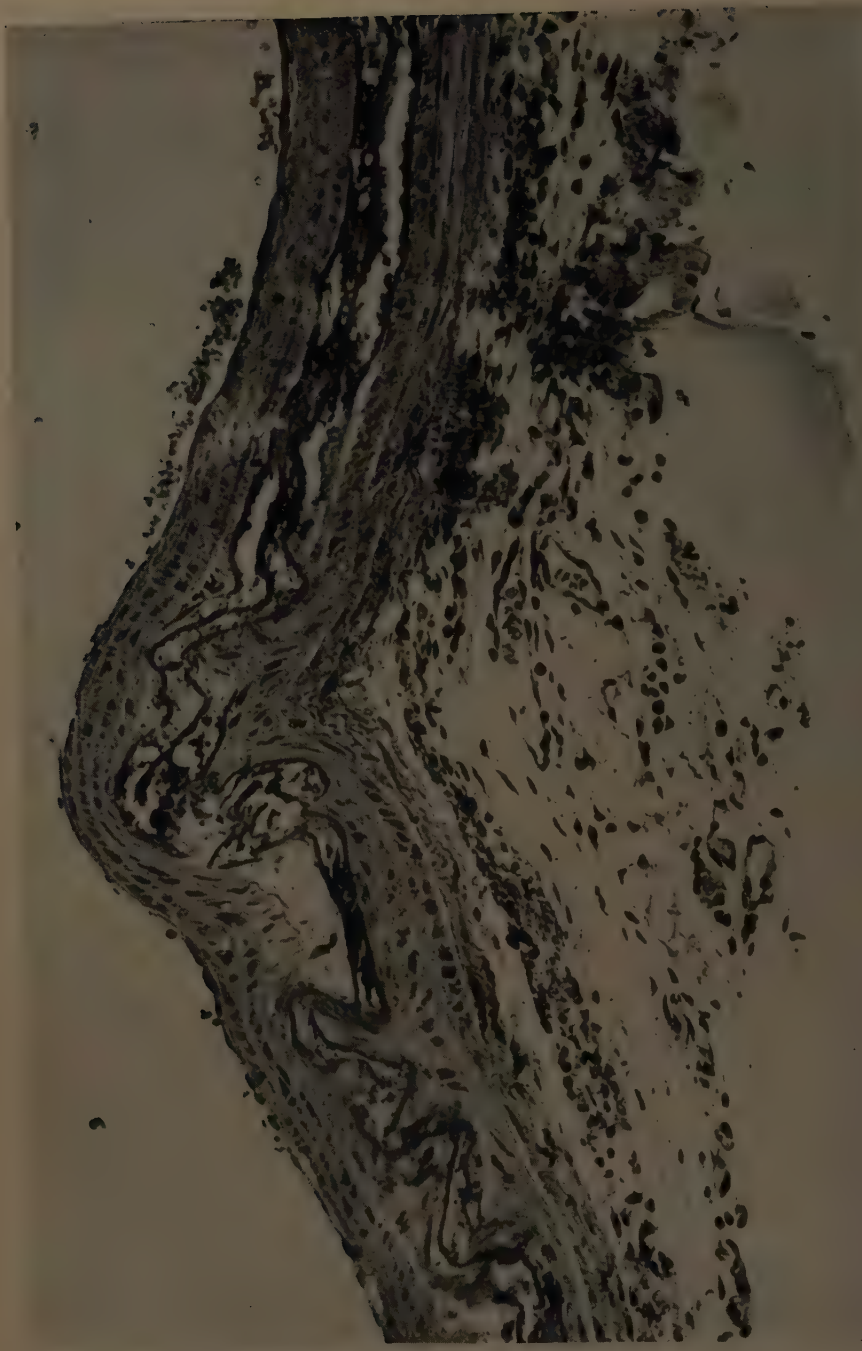

FIGURE 6. Aorta of rat 16 days after renal injury. Advanced destruction of aortic media with complete loss of normal pattern in affected area, kinking and extensive calcium imbibition of elastic membranes, and fatty metamorphosis of necrotic muscular debris. There is some infiltration of the periadventitial tissue with mononuclear cells (upper right part of the photograph). Hematoxylin-eosin stain, medium magnification.

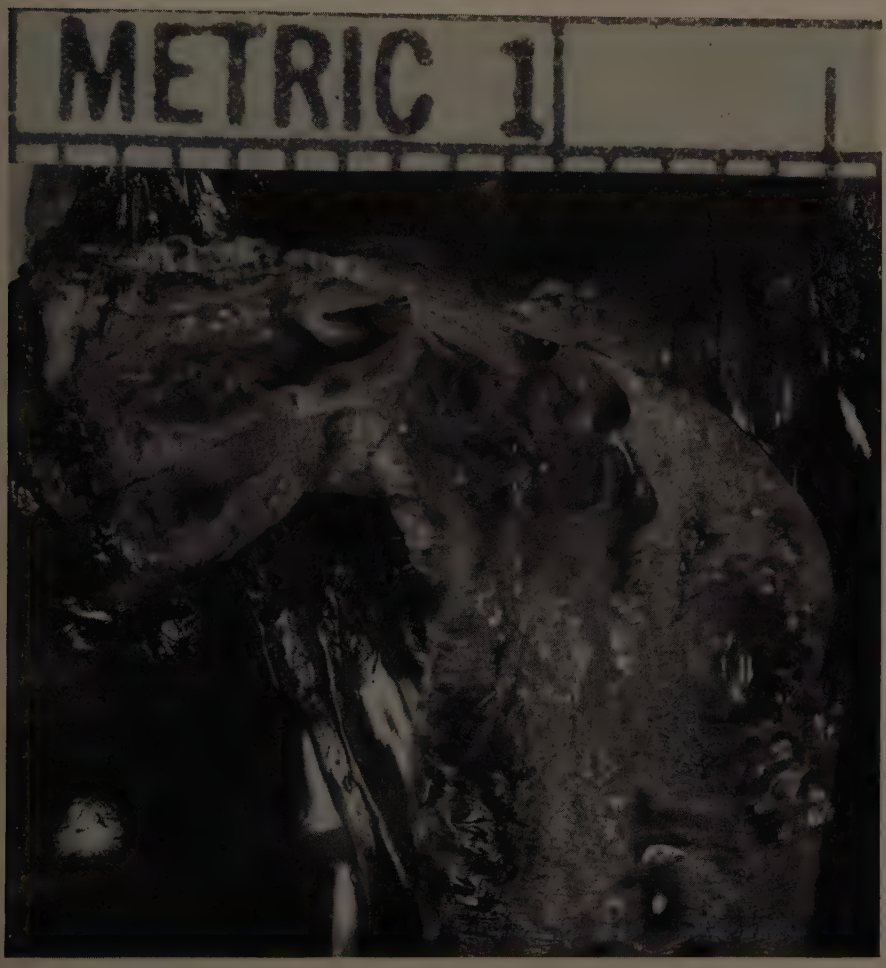

FIGURE 7. Opened aortic arch of rat 14 days after renal injury. "Tree-bark" appearance of intimal surface, with multiple aneurysmic dilations of the severely damaged vessel wall.[11] Reproduced by permission from *Endocrinology*.

"goose's-trachea" or "bamboo-stick" appearance, a picture that is well known in the human medial sclerosis of Mönckeberg. Both types of lesions were sometimes seen in different regions of the same aorta (FIGURE 12).

Heart. The typical alterations appearing within 24 hours consisted in diffuse and focal infiltrations of the myocardium, mostly by mononuclear cells (FIGURE 13), followed on the second day by edema of the myocardium and additional infiltration with polymorphonuclear leukocytes. Within 2 to 3 days, variation in staining intensity and coloring of muscle fibers indicated areas of degeneration involving either a segment of a single muscle fiber or a group of adjoining fibers. By the fourth day the affected fibers appeared swollen, became more

FIGURE 8. Translucency photograph of opened arch and thoracic aorta of the rat, showing extreme destruction of the wall 10 days after renal injury. Note the extensive irregular thinning (saccular aneurysm) of the wall and the widening of its circumference.

acidophilic, lost their nuclei and characteristic striation, began to rupture and, finally, to disintegrate, often leaving only a fine network of stroma. No conspicuous inflammatory reaction was found in these small areas of focal necrosis. In some instances larger areas of necrosis were noted involving a considerable portion of the myocardium, with marked acute inflammatory response. In such cases neutrophil and mononuclear cells were seen to surround and to invade the muscular debris. The affected muscle fibers or bundles may turn basophilic and undergo intensive calcification; they may then remain visible for weeks following the renal injury. Other areas may heal by fibroblastic proliferation, which starts after about 7 to 10 days and results in the formation of small scars (FIGURES 14, 15, and 16).

Larger areas of intensive cellular infiltration or of muscular necrosis and calcification were distinctly visible to the naked eye as yellowish, grayish white, or chalky white patches, slightly elevated above the normal surrounding surface of the myocardium and often permeating the entire myocardial cross section as a network of streaks and dots of the same colors clearly offset against the red of the healthy myocardium ("tigering," see FIGURE 17).

Gastrointestinal tract. As in the heart, the primary injury consisted again in degeneration and necrosis of the musculature. The site of predilection of this lesion was the thick muscular coat of the pyloric half of the rat stomach.

Under the microscope, lesions were seen by the second and third day following renal injury. They consisted in swelling and increased acidophilia of smooth muscle fibers, followed by pyknosis, loss of nuclei and of myofibril

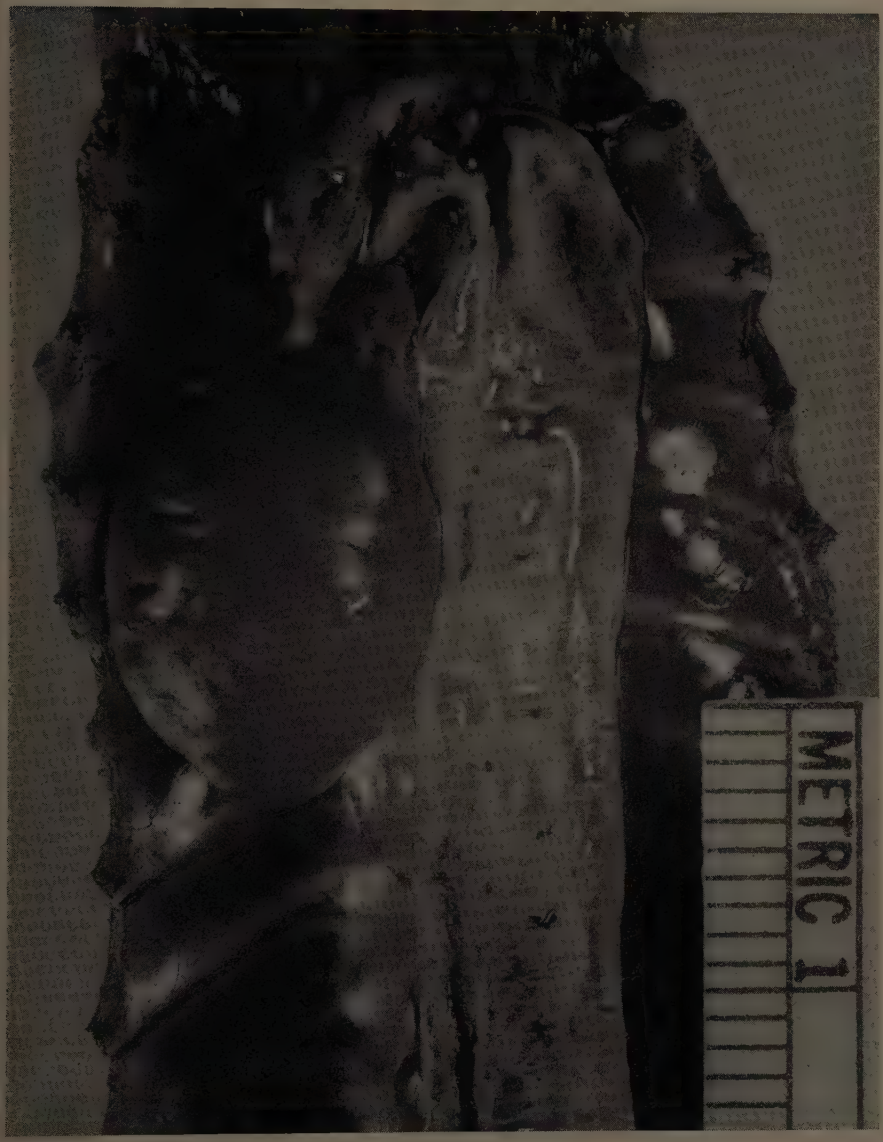

FIGURE 9. Heart and thoracic aorta of the rat (same animal as in FIGURE 7). The aneurysmic arch (not yet opened) has collapsed. Note the severe tree-bark appearance, especially of the abdominal intimal surface and the widening of the diameter to about twice normal.[11] Reproduced by permission from *Endocrinology*.

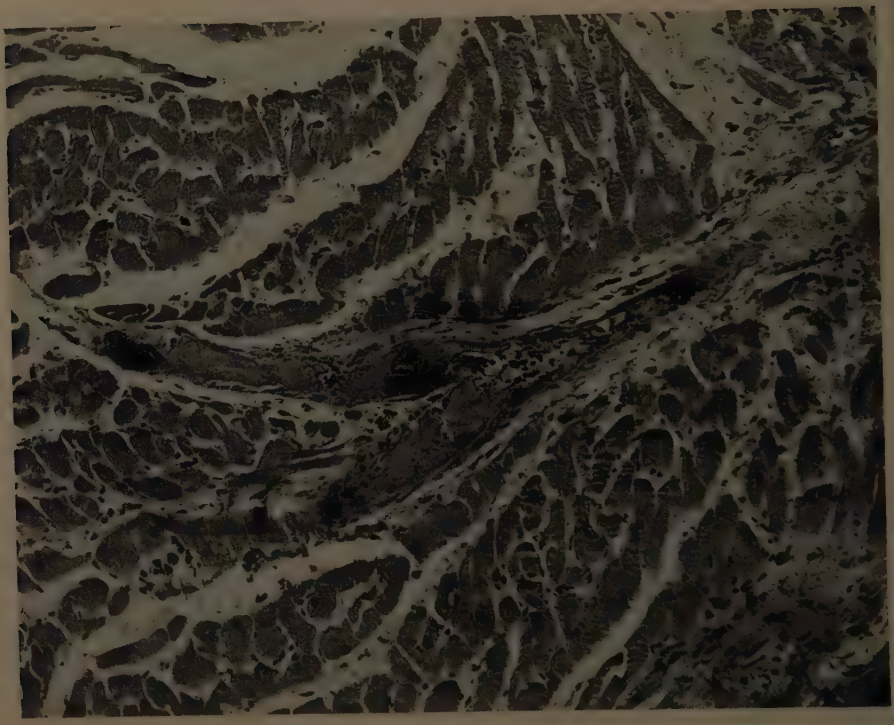

FIGURE 10. Myocardium of the rat 7 days after renal injury. Oblique and longitudinal section through branching arteriole of coronary system, showing sectional calcium imbibition of necrotic media (center and left margin of photograph). Hematoxylin-eosin stain, high magnification.

structure and, finally, necrosis and partial disappearance of the cytoplasm of muscle cells. This change occurred most characteristically in the central area of the powerful circular muscle bundles which, as in the myocardium, were replaced by a loose reticulum of stroma (FIGURE 18). There was little or moderate inflammatory response evidenced, as in the heart, by infiltration with mononuclear cells and polymorphonuclear leukocytes. Calcium imbibition of necrotic areas started at about the same time and increased in intensity in the second and third weeks (FIGURE 19). Some deposition of calcium salts was seen also in the mucosal lining of the stomach (FIGURE 20), which often manifested, in addition, multiple flat ulcerations.

To the naked eye the areas of cellular infiltration and necrosis appeared on the surface of the stomach as grayish white, often elevated streaks and dots that diverged in fanlike fashion from the small curvature, circling in the thick muscular coat toward the greater curvature and, in severe instances, engulfing the entire circumference like a spider web. Calcification of these necrotic muscle bundles resulted in the picture of chalky white ridges that seemed to follow the course of branching gastric vessels on the ventral and dorsal surface

of the stomach (FIGURE 21). Lesions of similar nature were seen, although less frequently, in the small and large intestines (FIGURE 22).

Hormonal Mechanisms and Interrelations

From the very inception of this work, hormonal mechanisms were believed to be prominently implicated in the development of disseminated muscular necrosis and calcification. In 1943, when I first published a standardized method for the production of renogenic arterial necrosis and calcification,[6] I stated that "special attention would be devoted to the relationship of changes in endocrine organs, in particular the thyroid, the parathyroid, pituitary and adrenals, and the arterial damage." The following changes, indicative of increased activity, were noted at that time in the parathyroid gland: "The entire gland appeared enlarged. The cells were increased in size and contained large, markedly vesicular nuclei, surrounded by considerable amounts of granular eosinophilic cytoplasm. In such instances the matrix of the tracheal cartilage was distinctly less basophilic, sometimes even frankly eosinophilic."

Subsequently these early histological findings were amply confirmed and expanded, and it was demonstrated that disseminated muscular necrosis could

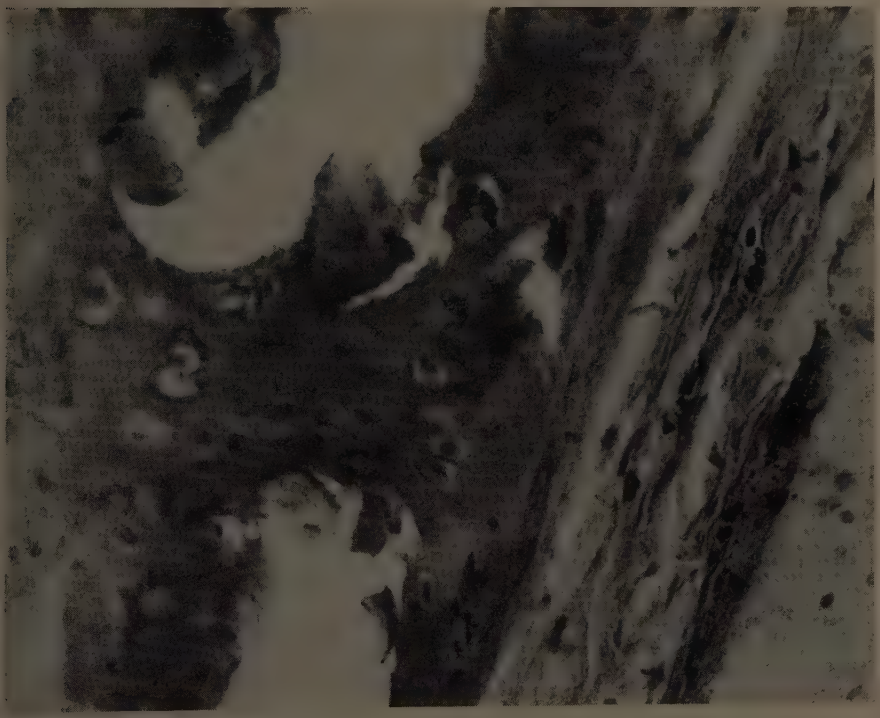

FIGURE 11. Aorta of the rat 6 weeks after renal injury. Bony metaplasia of calcified aortic ring, replacing entire width of media. At right, aortic adventitia. Hematoxylin-eosin stain, high magnification.

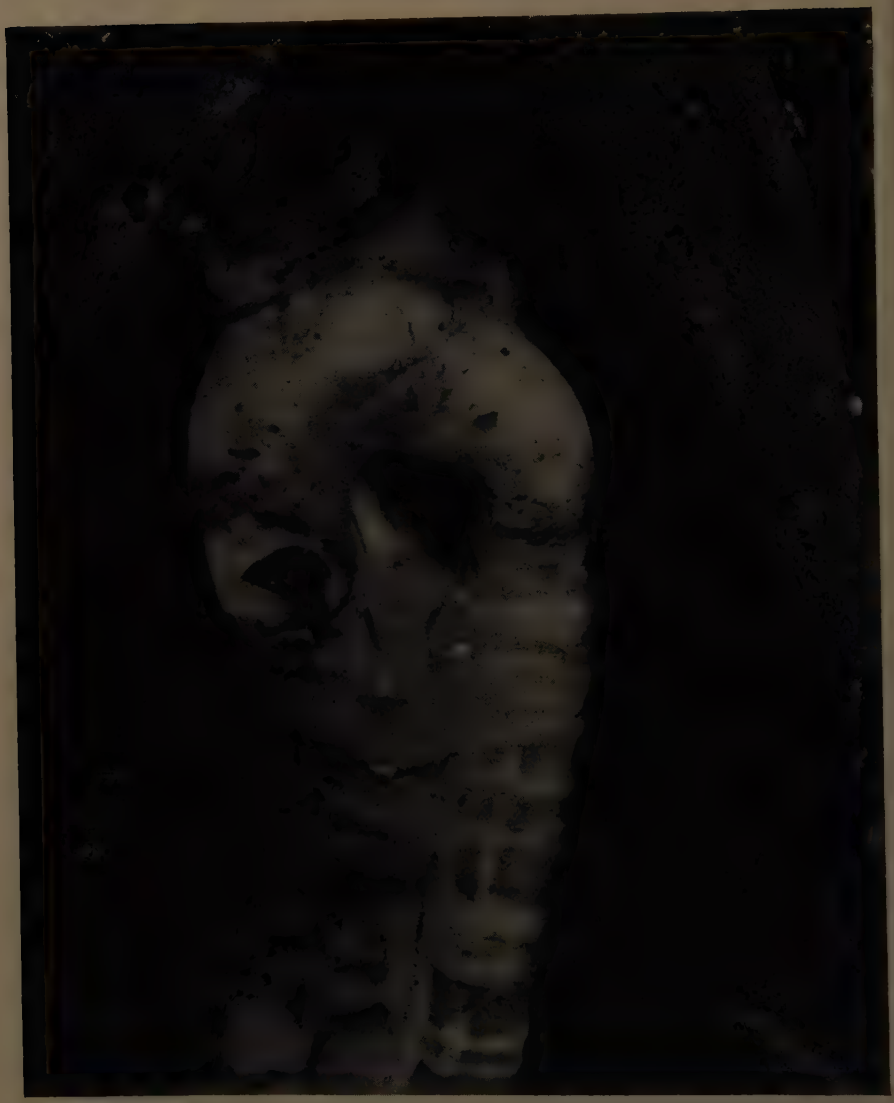

FIGURE 12. Large vessels of the neck, aortic arch, and thoracic aorta of the rat 4 weeks after standard renal injury. Note the combination of "pipestem" and "goose's-trachea" lesions in the same specimen.

not be produced by means of renal damage in thyroparathyroidectomized (TPx) rats.[11]

TABLE 1 exemplifies the high incidence of cardiovascular and smooth muscle necrosis in a group of 11 intact rats and the complete absence of necrosis at the same sites in 13 TPx rats, although renal injury was equally severe in intact and TPx animals, except for the absence of calcinosis in the latter group.

The influence of thyroid and parathyroid hormones. Further analysis of the remarkable protective effect of TPx (TABLE 1 and TABLE 2, Group 2) was directed toward elucidation of the relative importance of the separate thyroid and parathyroid hormones (TABLE 2, Groups 3 to 11). With the help of elective surgical removal and hormone substitution as well as "chemical thyroidectomy" and, finally, hormone excess, the influence of the following six conditions upon the incidence and severity of organic lesions was investigated: (1) adequacy of hormone substitution in TPx rats ("C"); (2) parathyroidectomy (Px) alone; (3) thyroidectomy (Tx) alone (TPx with parathyroid hormone replacement); (4) parathyroid deficiency in the absence of the thyroid (TxP−); (5) severe thyroid deficiency (chemical thyroidectomy, T−); and (6) excess thyroid or parathyroid hormone or both in intact rats (CT, CP, and CTP).

The results of these studies are summarized in TABLES 2, 3, and 4. TABLE 2 depicts the incidence of muscular necrosis, using, for the sake of conciseness, the most important and consistent lesion (arterial necrosis) as representative of events at all predilection sites. It is apparent from TABLE 2 that, in the absence or deficiency of the parathyroid hormone (Groups 2, 4, and 7), medionecrosis does not occur in the arterial tree, irrespective of the presence or ab-

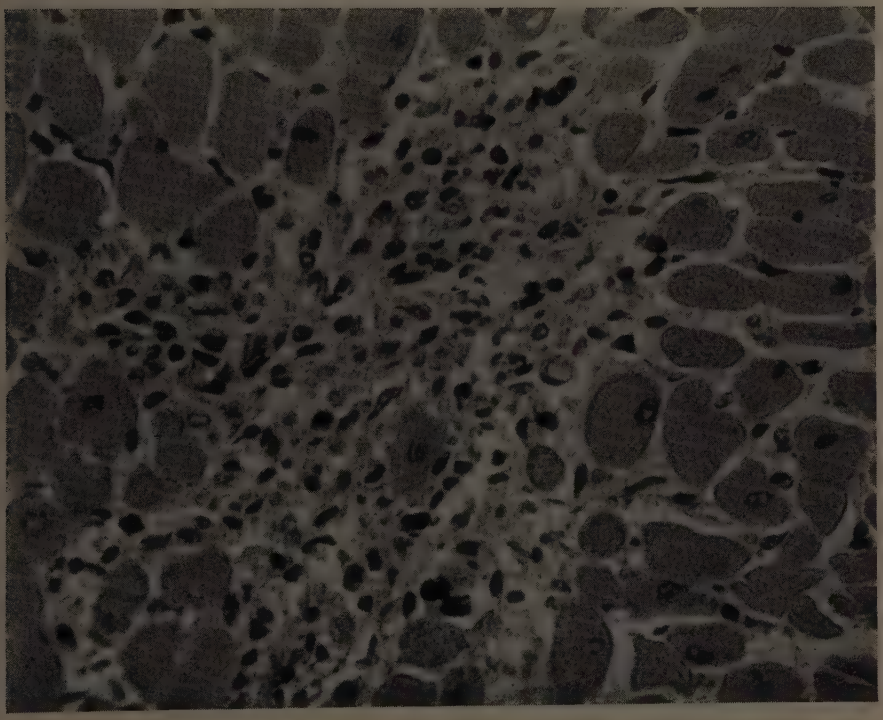

FIGURE 13. Myocardium of the rat 2 days after renal injury. Focal infiltration with mononuclear cells and disappearance of muscle fibers, leaving a syncytium of muscular stroma. Note the single intact muscle fiber in the lower center of the photograph, surrounded by the cellular infiltrate. Hematoxylin-eosin stain, high magnification.

FIGURE 14. Myocardium of the rat 5 days after renal injury. The area illustrated is part of a papillary muscle showing, on the left, normal muscle fibers and, in the center, hydropic degeneration and necrosis of muscle cells with loss of myofibril structure, disappearance of nuclei, and changes in staining characteristic. The collection of fibers in the top center of the photograph had become intensely basophilic. The right portion of the picture illustrates replacement of muscle fibers over a wide area by young connective tissue as an expression of organization and repair of the damage. The proliferation of histiocytes and the formation of new capillaries is clearly apparent. Hematoxylin-eosin stain, medium magnification.

sence of the thyroid hormone. There is also no calcium deposition in the damaged kidneys of Px rats. These findings are based on a total of 46 rats deprived of their parathyroid glands (Groups 2, 4, and 5) and are in sharp contrast to the findings in the 28 intact rats of this series (Group 1) and 200 additional intact rats from other experiments (not illustrated), all of which had nephrocalcinosis and most of which showed the typical lesions in the arterial tree.

Absence of the thyroid hormone, on the other hand, had no protective effect against muscular necrosis (Group 6), whereas simultaneous reduction of the parathyroid hormone supply to 20 units daily and less resulted in the prevention of the arterial medionecrosis, although these amounts were still adequate for the production of slight nephrocalcinosis (Group 7).

"Chemical thyroidectomy" (Group 8) appeared to have an inhibiting influence both on incidence and severity of arterial medionecrosis; this is in

confirmation of many of our previous studies using the thiouracils (unpublished data). Since the complete absence of thyroid hormone did not interfere with the development of severe muscular necrosis as long as the presence of adequate concentrations of parathyroid hormone was assured, the inhibition of the typical alterations by propylthiouracil administration was believed to be due to reduction of parathyroid activity as a consequence of thyroid deficiency.

In this connection it is of interest to note that excessive amounts of thyroid hormone produce marked aggravation of muscular necrosis and calcification and a definite acceleration in the emergence of the characteristic lesions in intact rats with renal block (Group 9). Similar aggravation was achieved in such animals also by the administration of parathyroid or of both thyroid and parathyroid hormones (Groups 10 and 11). It should be added that, in the dosages used, neither of these hormones nor their combination had any noticeable deleterious effect upon the organs of intact rats without renal injury.

On the basis of these findings it was inferred that the effects of the thyroid hormone are mediated through its direct or indirect action upon the para-

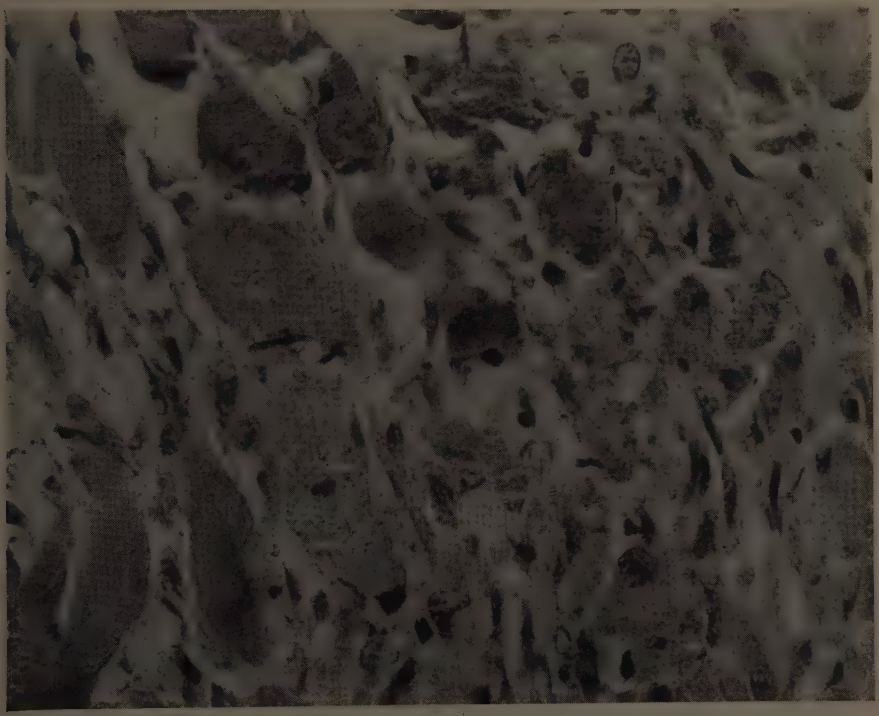

FIGURE 15. Myocardium of the rat 5 days after renal injury. View of approximate center of preceding picture (FIGURE 14) to illustrate in greater detail the destruction of muscle cells and the invasion of histiocytes. Note the still normal muscle cells at the left margin, the hydropic degeneration and basophilic staining in the center, and the complete disintegration of the necrotic muscle fibers at the right side of the photograph. Hematoxylin-eosin stain, high magnification.

thyroid gland, thyroid deficiency causing simultaneous reduction of parathyroid function and excess thyroid acting as a stimulus of parathyroid activity.

Further support for this point of view will become apparent as some of the "clinical" data are subjected to analysis.

Following renal injury, rats of all groups manifest a similar transient rise in plasma total cholesterol and marked reduction of its carbon dioxide content as signs of impairment of kidney function. They also uniformly have oliguria or anuria during the first 24 hours. The onset of polyuria occurs within the next 24 hours; individual rats may reach a daily urine volume of 80 ml. No significant differences in urine volume were noted between the various experimental groups except for a marked preblock water diuresis in the groups receiving considerable doses of parathyroid extract.[3, 6, 10, 11]

TABLE 3 illustrates the behavior of the systolic blood pressure. Intact rats (Group 1) follow the pattern depicted in FIGURE 1; namely, a rise in systolic pressure starting at about the fourth day after renal block and continuing into the second week, followed by gradual return toward preblock levels.

In all animals in which there is an absence or deficiency of the thyroid hormone (Groups 2, 6, 7, and 8), mean systolic blood pressures remain within

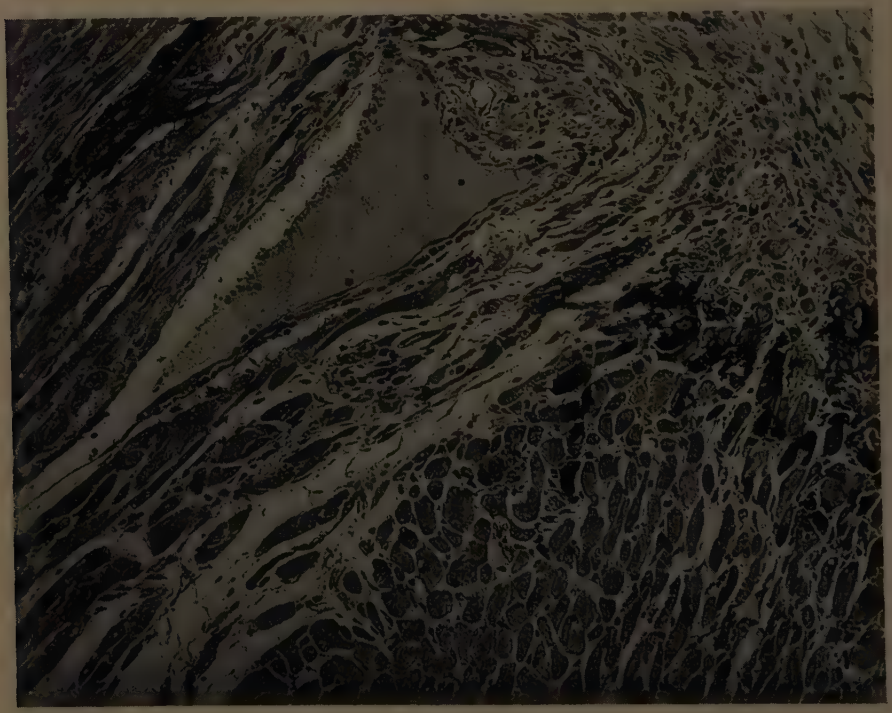

FIGURE 16. Myocardium of the rat 10 days after renal injury. Intense calcification of individual muscle fibers. The remainder of myocardial structure appears essentially normal. Hematoxylin-eosin stain, medium magnification.

the normal range after renal injury, whereas there is definite early hypertension in all thyroid-treated rats (Groups 3, 4, 9, and 11) irrespective of the presence or absence of the parathyroid hormone.

Absence of the parathyroid only did not prevent the emergence of hyper-

FIGURE 17. Close-up view of rat heart 5 days after standard renal injury. Note the patchy necrosis of the myocardium, represented in this photograph by the light gray areas. Dissemination throughout the myocardium produced a "tigering" effect. In order to eliminate disturbing highlights, the heart was photographed through a liquid barrier. Saline was employed rather than glycerol to preserve the integrity of the specimen.

tension although, during the initial 3 days after renal block, blood pressure readings dipped to the low range of the normal (Group 5).

Exogenous parathyroid failed to induce hypertension in TPx rats (Group 6) but, when given in the presence of either endogenous or injected thyroid hormone (Groups 3, 10, and 11), it seemed to hasten the onset of hypertension and possibly also to enhance its degree.

The data depicted in TABLES 2 and 3 permit the following three deductions: (1) the emergence of hypertension secondary to obstructive nephropathy is dependent upon the presence of adequate amounts of injected or endogenous thyroid hormone; (2) hypertension per se does not necessarily produce vascular necrosis under our experimental conditions (Group 4); and (3) hypertension is not required for the emergence of characteristic lesions (Group 6).

The effects of alterations in thyroid and parathyroid activity upon the plasma levels of calcium and inorganic phosphate are summarized in TABLE 4. Before the production of renal injury (preblock) these levels show the following behavior under the influence of the various experimental procedures.

Intact controls have a mean plasma concentration of 10.9 mg. per cent calcium and 3 mg. per cent inorganic phosphate. Surgical parathyroidectomy,

FIGURE 18. Muscularis of the rat stomach 5 days after renal injury. Loose reticulum of stroma replacing muscle bundles in central portions of circular muscle coat. There is some infiltration of the muscularis with mononuclear cells. Hematoxylin-eosin stain, medium magnification.

with or without simultaneous removal of the thyroid glands (Groups 2, 4, and 5), brought about the expected fall in calcium and rise in phosphate level. In TPx rats the administration of parathyroid extract caused a pronounced rise in calcium when given alone (Group 6), but when the same dose was combined with thyroid administration (Group 3), an elevation of the phosphate level occurred instead.

In line with this observation, although preliminary because of the small number of rats involved, parathyroid extract administered to intact animals raised the initial plasma calcium level to 14.0 mg. per cent when administered alone (Group 10), and to 12.3 mg. per cent when administered in combination with thyroid extract (Group 11). Possible explanations for an antagonistic effect of the thyroid hormone upon calcium plasma levels may lie in a more rapid elimination of calcium from the body or in a speedier metabolism of injected parathyroid in the presence of thyroid hormone. The abnormally high calcium levels in intact rats under the influence of excess parathyroid hormone (Groups 10 and 11) are accompanied by high phosphate levels, possibly because mobilization of calcium phosphate from the skeleton exceeds the increased tubular elimination of phosphate under the influence of parathyroid hormone.

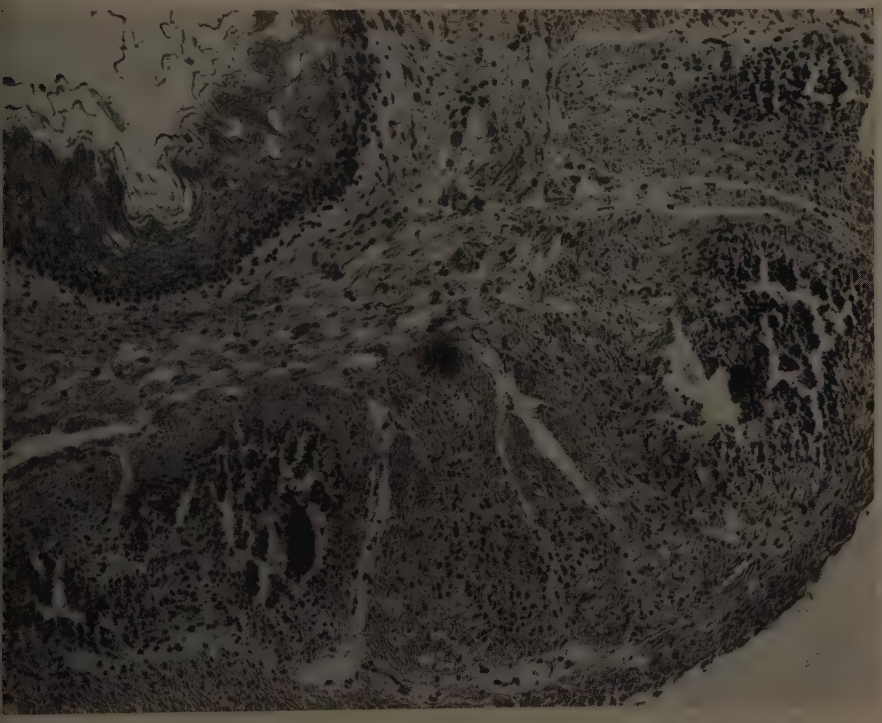

FIGURE 19. Stomach of the rat 10 days after renal injury. Necrosis and calcification of central portions of circular muscle bundles and moderate infiltration of muscularis with mononuclear cells and some polymorphonuclear leukocytes. Hematoxylin-eosin stain, medium magnification.

Following renal block there is definite elevation of the calcium concentration in all animals with intact parathyroid glands (Groups 1, 8, and 9), also in those with adequate parathyroid substitution (Groups 3 and 6) and, finally, in those with excess of this hormone (Groups 10 and 11). Simultaneously there occurs a further fall in the calcium level of rats deprived of the parathyroid glands (Groups 2 and 5) and also in those inadequately substituted with parathyroid

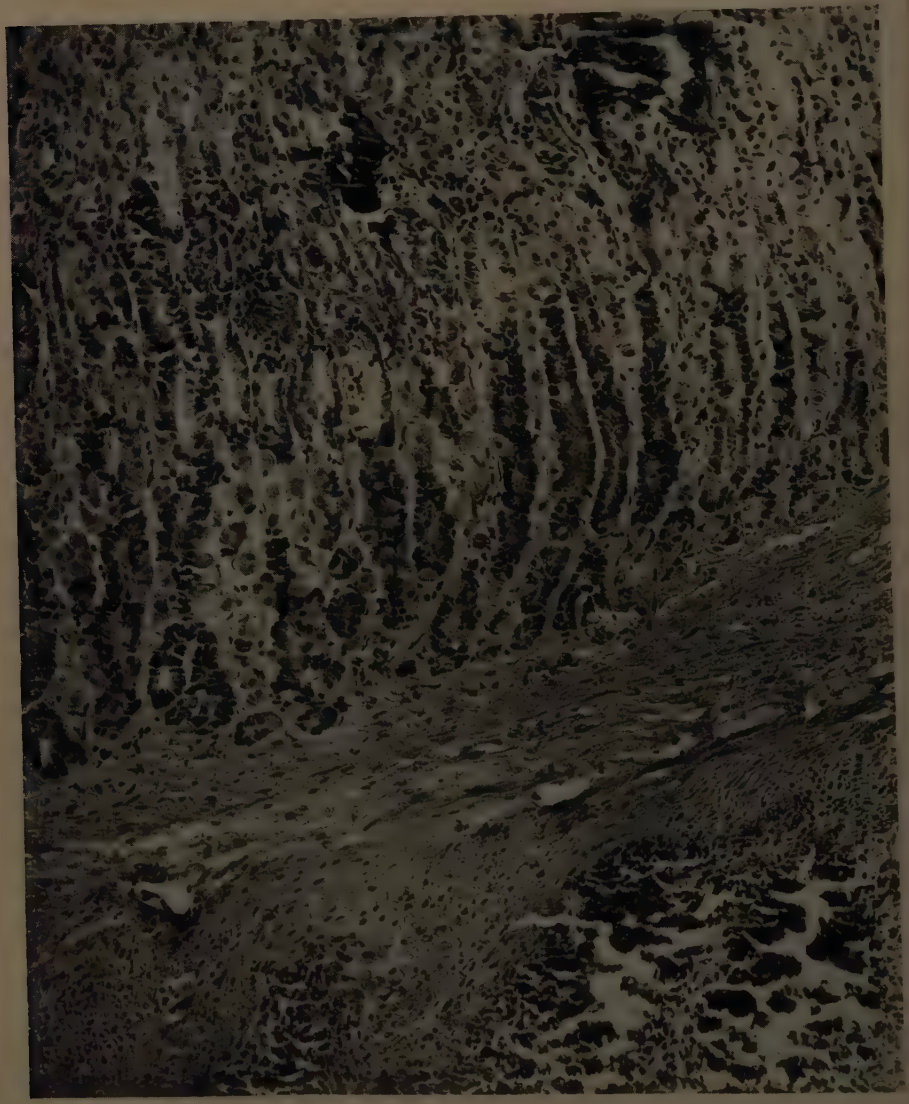

FIGURE 20. Stomach of the rat 7 days after renal injury. *Top*, calcium deposits in 2 areas of the mucosal lining; *bottom*, necrosis and calcification of the muscularis as shown in FIGURE 19. Hematoxylin-eosin stain, medium magnification.

(Group 7). On the whole, these changes in the plasma calcium level appear to be maintained for about 2 to 3 weeks.

It is of particular interest that animals receiving propylthiouracil (Group 8) have somewhat low initial calcium levels, while thyroid-treated intact rats are hypercalcemic if compared with the controls (Group 9). In fact, a remarkable elevation of the calcium level was recorded in Group 9 on the second day after renal injury, which once more suggests an enhancing effect of thyroid hormone upon the parathyroid gland. This group, although small, is of particular interest, since each of the 3 rats manifested the severest degrees of nephrocalcinosis ever observed in our experiments and had the largest parathyroid glands (several times the normal size). However, direct mobilization of calcium from the depots of the body by thyroid hormone must also be considered since, in the complete absence of parathyroid hormone, thyroid substitution alone was able to maintain calcium levels well within the normal range following renal injury in the face of pronounced phosphate retention (Group 4).

Comparison of appearance and of moist weights of organs from animals in the

FIGURE 21. Stomach of the rat 16 days after standard renal injury. The pyloric part of the stomach is distinguished by a thick muscular coat. Note that this part, in particular, contains numerous calcified ridges that converge in fanlike fashion toward the small curvature.

various experimental groups revealed that the administration of thyroid caused a very substantial increase in the size and weight of the adrenals and the heart and a lessening of thymic atrophy following renal injury. This applied to intact as well as TPx rats.

From the summary of results presented in TABLE 2, it appears established that muscular necrosis does not develop in the absence of the parathyroid hormone, whereas the absence of thyroid hormone does not prevent the development of typical pathologic-anatomical changes.

The role of adrenal cortical hormones. The essential results of studies with adrenalectomized (Ax) rats are summarized in TABLE 5, Groups 6 to 9. For the sake of clarity and ease of comparison, a condensation of pertinent data on the influence of the thyroid and parathyroid hormone on muscular necrosis discussed in the preceding section (including additional confirmatory experiments) is presented in Groups 1 to 5 of TABLE 5.

The singular significance of the parathyroid hormone in the pathogenesis of arterial medionecrosis is dramatically emphasized by the complete absence of this lesion in 30 Px rats (Group 4). Moreover, one can readily enlarge this number by adding the 14 animals of group 8 and, since suppression of thyroid

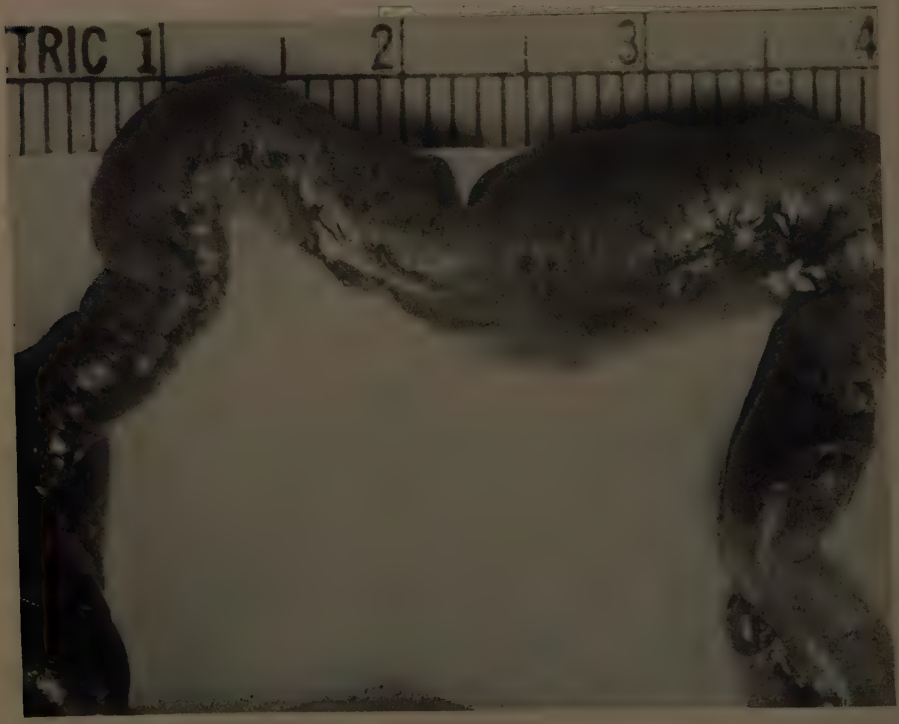

FIGURE 22. Colon of the rat 15 days after standard renal injury. Extensive patchy necrosis of muscularis, especially of transverse colon, resulting in a weblike pattern of slightly elevated grayish ridges on the surface of the gut.

Table 1

Production and Prevention of Muscular Necrosis*

Organic Changes in 11 Intact and 13 Thyroparathyroidectomized (TPx) Rats

Organ	Pathologic-anatomical changes				
	Type	Number and incidence			
		Intact group		TPx group	
		No.	Per cent	No.	Per cent
Heart	Focal myocardial necrosis and/or diffuse and focal infiltrations with mononuclear cells (myocarditis)	11	100	2†	15
Coronary arteries	Medionecrosis and calcification	10	91	0	0
Aorta and branches	Medionecrosis and calcification	9	82	0	0
Stomach	Necrosis and calcification of muscularis	9	82	0	0
Kidneys	Parenchymatous degeneration, pyelonephritis, interstitial nephritis	11	100	13‡	100

* Modified from Lehr and Martin (Table 2).[11]
† Myocarditis only.
‡ No nephrocalcinosis in any animal of this group.

activity fails to obviate muscular necrosis (Group 3), also the 35 rats of Group 2.

Thus, none of a total of 79 parathyroprive rats showed any vascular lesion or other muscular necrosis under careful microscopic examination.

Administration of exogenous parathyroid, on the other hand, fully restored the ability of such animals to develop characteristic lesions (Group 5). Thus, of 18 Px rats in Group 5 receiving exogenous parathyroid by injection, 13 (72 per cent) manifested typical muscular necrosis at the usual sites.

The role of the adrenal cortex in the mechanism of this experimental cardiovascular disease could not be fully explored, since adrenalectomized rats maintained on saline were unable to survive the stress of the procedure required for the production of standard renal injury for more than a few hours.

Pretreatment with desoxycorticosterone acetate (DCA) failed to effect any substantial prolongation of life. The substitution of cortisone resulted in adequate lengthening of survival time, but without the appearance of parathyroid hypertrophy and without the development of cardiovascular necrosis (Group 6). On the other hand, pretreatment of adrenalectomized rats with both DCA and cortisone (Group 7) permitted satisfactory survival as well as enlargement of the parathyroid gland and the appearance of severe cardiovascular necrosis.[23] Of 20 animals in Group 7, 12 (60 per cent) showed the typical alterations. However, when the same combination of mineralocorticoid and glucocorticoid in identical dosage was given to rats deprived of their parathyroid glands in addition to their adrenals, no muscular necrosis could be produced by renal injury (Group 8), whereas adrenalectomized rats maintained on cortisone and receiving parathyroid extract developed a high incidence of typical injury in the arterial tree and other predilection sites (Group 9). Thus,

TABLE 2

PRODUCTION OF CARDIOVASCULAR NECROSIS IN THE RAT

The Influence of Thyroid and Parathyroid Activity upon the Development of Arterial Medionecrosis Following Renal Injury

Group No.	Glands surg. removed	Hormone treatment or block	Physiological state		Total No. of rats	No. of rats with organic damage		
			Definition	Abbrev. symbol		Kidney		Aorta
						Obstruct. nephropathy	Tubular calcif	Medionecrosis
1	None	None	Intact animal	C	28	28	28	23
2	Thyroid Parathyr.	None	Thyroparathyroidectomy	TPx	24	24	0	0
3	Thyroid Parathyr.	Thyroid* Parathyr.†	Intact animal	"C"	5	4	2	2
4	Thyroid Parathyr.	Thyroid*	Parathyroidectomy	Px	12	9	0	0
5	Parathyr.	None	Parathyroidectomy	Px	10	8	0	0
6	Thyroid Parathyr.	Parathyr.†	Thyroidectomy	Tx	6	6	6	5
7	Thyroid Parathyr.	Parathyr. (10–20 units only!)	Thyroidectomy Parathyroid deficiency	TxP−	5	5	2 (trace)	0
8	None	Propylthiouracil‡	Hypothyroid Hypoparathyroid?	T− P−?	10	9	8	2
9	None	Thyroid*	Hyperthyroid	CT	3	3	3	2
10	None	Parathyr.†	Hyperparathyroid	CP	3	3	3	3
11	None	Thyroid* Parathyr.†	Hyperthyroid and hyperparathyroid	CTP	3	3	3	3

* Thyroid powder, 2.5 mg. } daily S.C., 5 days preceding and 5 days following renal
† Parathyroid extract, 50 units } block.
‡ Starting 4 weeks prior to renal block, 0.01 per cent was used in the drinking water.

while DCA was essential for the development of cardiovascular necrosis in the presence of the animal's own parathyroid gland, this injury could be readily produced in the absence of DCA by excess parathyroid hormone. Consequently, it would seem that under the conditions of our experiments DCA itself is not injurious, but that an ample supply of this or a similar corticosteroid is essential for the production or release of excess parathyroid hormone, which in turn is responsible for the development of disseminated muscular necrosis. Hence, a direct or indirect stimulatory action of mineralocorticoids upon the parathyroid gland must be postulated.

On the basis of the evidence derived from our studies the mechanism leading to cardiovascular and smooth muscle necrosis following renal injury is believed to encompass the following events.

Severe impairment of renal function (obstructive nephropathy) and the resulting pronounced derangement of the mineral metabolism cause stimulation of the parathyroid glands and of the adrenal cortex. The increased output of mineralocorticoids triggers the release of excess parathyroid hormone which, in

turn, is responsible for disseminated muscular necrosis and calcification and further aggravates the renal injury (nephrocalcinosis). Thus a vicious circle is established (FIGURE 23).

Because the necrosis occurs in the presence of abnormally high calcium and inorganic phosphate levels in the blood, the deposition of calcium phosphate in the soft tissues may be implicated as the cause rather than as a consequence of necrosis. However, histological evidence of necrosis precedes any histologically demonstrable deposition of calcium salts in the affected soft tissues.

RENIPRIVAL CARDIOVASCULAR DISEASE

In the first part of this paper it was demonstrated that cardiovascular and smooth muscle necrosis induced in the albino rat by means of standard renal injury is due to the effect of excess parathyroid hormone released by the animal's own glands as a consequence of impaired renal function.

While these findings established the primary importance of an extrarenal mechanism in the production of muscular necrosis, they could not fully exclude the possible contributory effect of a renal "toxin."

Activation of the parathyroid glands to excess function was explained as due

TABLE 3

PRODUCTION OF CARDIOVASCULAR NECROSIS IN THE RAT

The Influence of Thyroid and Parathyroid Activity upon Blood Pressure Changes Following Renal Injury

Group No.	No. of rats	Type of surgery and/or treatment		Physiol. state (see TABLE 2)	Mean systolic blood pressure in mm. Hg					
					Pre-block	2 days post-block	3 days post-block	4 days post-block	7 days post-block	2nd wk. post-block
1	28	Intact	None	C	106	105	115	172	180	128
2	24	Thyroparathyroidectomy	None	TPx	100	100	102	85	102	124
	5	Thyroparathyroidectomy	Thyroid* Parathyroid†	"C"	106	132	193	235	‡	
4	12	Thyroparathyroidectomy	Thyroid*	Px	109	114	153	187	132	166
5	10	Parathyroidectomy	None	Px	94	96	80	184	189	108
6	6	Thyroparathyroidectomy	Parathyroid†	Tx	98	100	116	129	94	96
7	5	Thyroparathyroidectomy	Parathyroid (10–20 units only!)	TxP−	95	93	—	—	76	109
8	10	Intact	Propylthiouracil‡	T− P−?	105	95	104	152	129	104
9	3	Intact	Thyroid*	CT	112	175	§			
10	2	Intact	Parathyroid†	CP	119	175	180	§		
11	2	Intact	Thyroid* Parathyroid†	CTP	110	165				

* Thyroid powder, 2.5 mg. } daily S.C., 5 days preceding and 5 days following renal
† Parathyroid extract, 50 units } block.
‡ Starting 4 weeks prior to renal block, 0.01 per cent was used in the drinking water.
§ Experiment terminated by death.

TABLE 4

PRODUCTION OF CARDIOVASCULAR NECROSIS IN THE RAT

The Influence of Thyroid and Parathyroid Activity upon Changes in Plasma Calcium and Phosphate Levels Following Renal Injury

Group No.	No. of rats	Type of surgery and/or treatment		Physiol. state (see TABLE 2)	Mean plasma levels in mg per cent; total calcium (Ca) and inorganic phosphate (P)*					
					Preblock		2 Days postblock		Terminal†	
					Ca	P	Ca	P	Ca	P
1	28	Intact	None	C	10.9	3.0	11.7	10.5	12.0	3.1
2	24	Thyroparathyroidectomy	None	TPx	9.0	5.6	7.6	8.4	8.3	6.2
3	5	Thyroparathyroidectomy	Thyroid‡ Parathyroid§	"C"	9.8	6.0	11.9	10.7	12.3	7.6
4	12	Thyroparathyroidectomy	Thyroid‡	Px	9.0	5.5	10.7	13.6	12.0	9.6
5	10	Parathyroidectomy	None	Px	9.0	7.2	8.2	11.6	9.4	5.9
6	6	Thyroparathyroidectomy	Parathyroid§	Tx	14.0	4.1	18.3	7.6	11.8	5.1
7	5	Thyroparathyroidectomy	Parathyroid (10–20 units only!)	TxP−	10.3	4.8	8.5	11.2	12.0	5.1
8	10	Intact	Propyl-Thiouracil‖	T− P−?	9.6	2.3	11.6	8.1	11.6	4.4
9	3	Intact	Thyroid‡	CT	11.5	2.2	17.2	9.0	9.6	9.3
10	2	Intact	Parathyroid§	CP	14.0	6.3	15.0	7.2	13.8	2.8
11	2	Intact	Thyroid‡ Parathyroid§	CTP	12.3	6.3	12.0	8.9	10.4	8.7

* Calculated as phosphorus.
† Usually 2 weeks, except groups 3, 9, 10 and 11 (see TABLE 3).
‡ Thyroid powder, 2.5 mg. } daily S.C., 5 days preceding and 5 days following renal
§ Parathyroid, extract 50 units } block.
‖ Starting 4 weeks prior to renal block, 0.01 per cent was used in the drinking water.

TABLE 5

PATHOGENESIS OF ARTERIAL MEDIONECROSIS INDUCED BY RENAL INJURY IN THE ALBINO RAT*

Group	Organs excised (x); thyroid—T; parathyroids—P; adrenals—A	Hormone substitution or excess	No. of rats	Arterial medionecrosis	
				No.	Incidence (percentages)
1	— (Intact controls)	—	46	43	93
2	TPx	—	35	0	0
3	"Tx"†	—	17	5	30
4	Px	—	30	0	0
5	Px	Parathyroid	18	13	72
6	Ax	Cortisone	24	0	0
7	Ax	Cortisone & DCA	20	12	60
8	Ax & Px	Cortisone & DCA	14	0	0
9	Ax	Cortisone & parathyroid	12	8	67

* Modified from Lehr and Martin (Table 1).[12]
† Chemical "thyroidectomy" (propylthiouracil block).

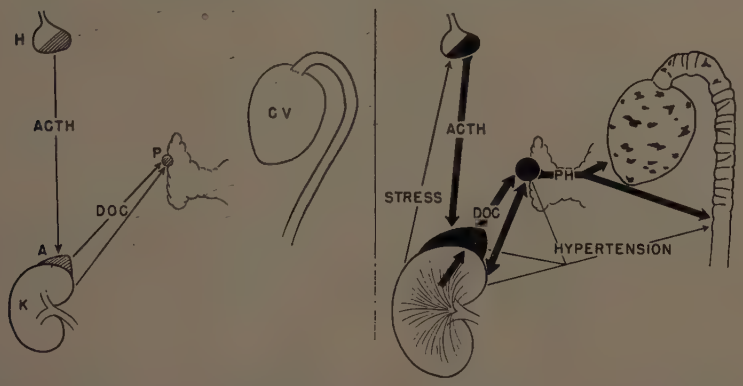

FIGURE 23. Schematic representation of the events leading to cardiovascular necrosis in the albino rat; *left*, normal control; *right*, after standard renal injury. Key: H, hypophysis; ACTH, adrenocorticotropic hormone; A, adrenal; CV, cardiovascular system; K, kidney; DOC, mineralocorticoid; PH, parathyroid hormone; P, parathyroid.

to disturbance of the mineral metabolism and increased production of adrenal corticoids resulting from the severe impairment of renal excretory function. It was reasoned, therefore, that if a renal toxin is not involved in the mechanism of cardiovascular necrosis, the outright removal of both kidneys might induce lesions comparable to those following standard renal injury by a similar mechanism and should thus be amenable to the same aggravating and extenuating influences. The validity of this reasoning was explored in studies with nephrectomized rats.

Signs Following Bilateral Nephrectomy

Recovery from the operation was complete within the first day. On the second and third days the rats were alert and active, drank some tap water, but ate little. There was only minor loss in body weight, usually not exceeding 5 per cent of the initial weight. Under some experimental conditions (administration of adrenal corticoids) waterlogging caused some gains in body weight. By the end of the third or fourth day the cumulative effect of mounting uremic poisoning caused rapid deterioration in the condition of the reniprival rat manifested by depression, difficulty in walking, tremors, weakness, labored respiration and, finally, death.

Studies of the behavior of calcium and phosphate in the serum and of the blood pressure after nephrectomy were carried out in special experiments in order not to interfere by added stress with the short life span and thus with the development of pathologic-anatomical changes in the main experiments.

Both calcium and inorganic phosphate levels rose above the physiological range within 1 hour following removal of the kidneys, reached concentrations near 20 mg. per cent for calcium as well as inorganic phosphate within 5 hours and climbed even higher within the first 24-hour period. TABLE 6 illustrates the values of 3 successive 24-hour periods.

It is apparent that calcium levels are maintained at approximately twice the normal range, whereas phosphate concentrations attain mean values of

TABLE 6
EFFECT OF NEPHRECTOMY (Nx) AND PARATHYROID EXTRACT ON CALCIUM (Ca) AND INORGANIC PHOSPHATE (P) LEVELS IN RAT SERUM

Time in hours after Nx	No. of rats	Nx		No. of rats	Nx + parathyroid*	
		Ca	P†		Ca	P†
24	5	25.0 ± 1.4	29.5 ± 4.0			
48	9	19.3 ± 6.7	34.0 ± 8.4	8	18.1 ± 4.2	34.0 ± 3.7
72	7	21.3 ± 4.6	9.1 ± 6.4	6	23.5 ± 4.1	43.0 ± 2.8

* Parathyroid extract (100 units), injected S.C. at 0, 24, and 48 hours.
† Calculated as phosphate.

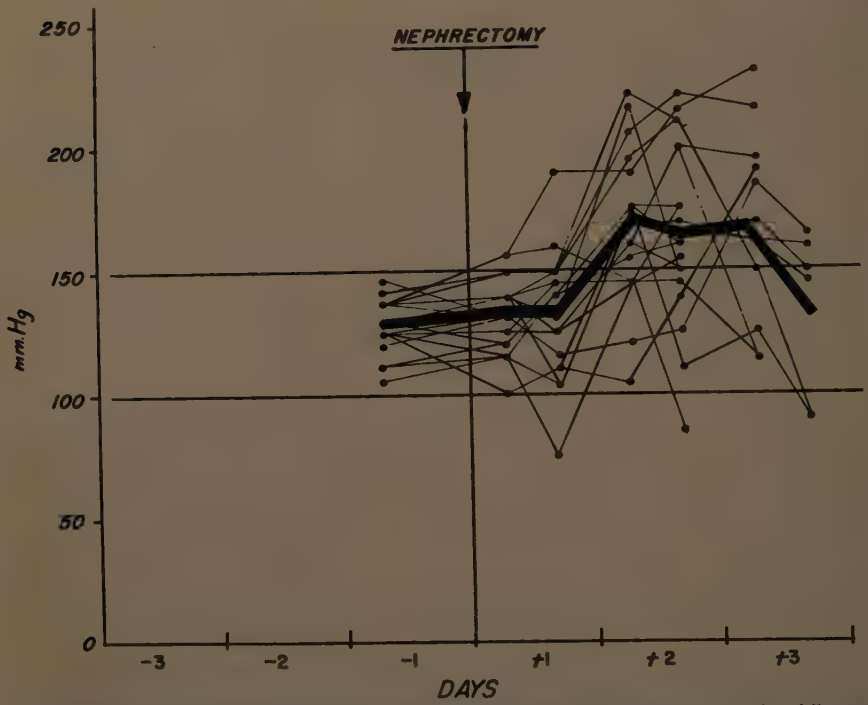

FIGURE 24. Individual systolic blood pressures of 20 albino rats before and after bilateral nephrectomy. Note the marked reniprival hypertension on the second and third days. The heavy black line represents the calculated mean from the values of all survivors.

34 mg. per cent in 48 hours and decline toward normal on the third day. The administration of additional exogenous parathyroid has no further influence on the calcium level, whereas the phosphate concentration reaches values of 43 mg. per cent on the third day under these conditions.

In agreement with others it was found that bilateral nephrectomy produces almost invariably considerable elevation of the systolic blood pressure of the rat.[24-26] Under the conditions of our experiments, hypertensive levels were recorded in some instances by the end of the first day and almost regularly at

the beginning of the second day following removal of both kidneys. Only readings above 150 mm. Hg were counted as hypertensive. The hypertension was usually maintained for the next 24 hours. On the third or, maximally, the fourth and final days of the reniprival life span the progressive deterioration caused a decline toward normotension and, finally, to shock levels. During the hypertensive phase systolic pressure readings ranged between 160 and 230 mm. Hg (FIGURE 24). It can be seen from this figure that, among 20 nephrectomized rats, at least 1, but usually 2 or more, marked hypertensive readings were registered during the reniprival life span of each animal. In fact, on the second or third day postnephrectomy 28 of 37 blood pressure measurements (76 per cent) were in the hypertensive range (155 to 230 mm. Hg), the rest remaining near the upper limit of the normal. The heavy black line indicates the mean of the values from the survivors at each point of observation. It is clear that this curve of mean values, which by necessity includes many low readings and shock levels of moribund animals, actually distorts the picture by de-emphasizing the intense hypertensive response seen in all rats that withstood bilateral nephrectomy well and proved more resilient against the added stress of 2 daily blood pressure recordings.

When the parathyroids were removed prior to nephrectomy, reniprival

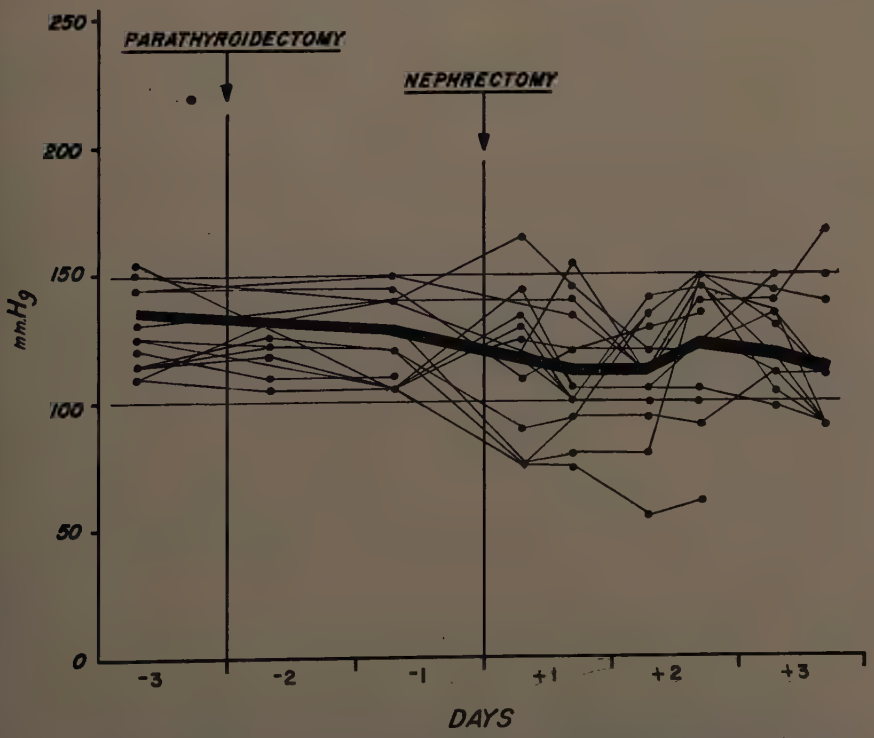

FIGURE 25. Individual systolic blood pressures of 15 albino rats before and after parathyroidectomy and nephrectomy. Note the complete absence of reniprival hypertension. The heavy black line represents the calculated mean from the values of all survivors.

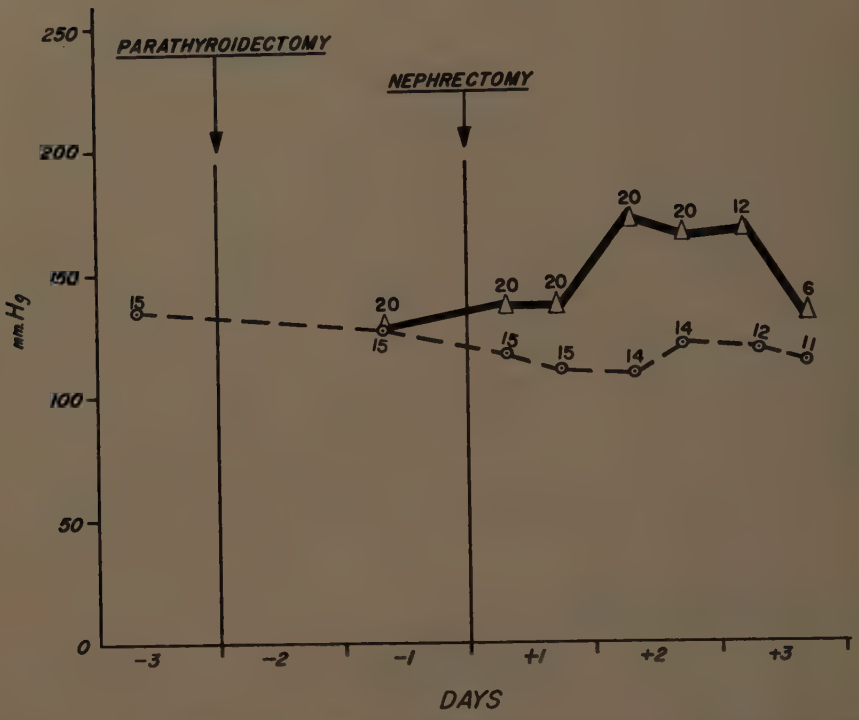

FIGURE 26. Comparison of the mean systolic blood pressures of intact and parathyroprival rats before and after bilateral nephrectomy. Note the striking difference in the blood pressure response of intact and parathyroprival rats to bilateral nephrectomy. The figures adjacent to the values represent the number of survivors available for measurement.

hypertension appeared to be abolished (FIGURE 25). Among 15 parathyroidectomized rats only 2 moderately elevated blood pressure readings were recorded: one on the first day and the other, on the third day postnephrectomy. All of 30 measurements taken on the crucial second day postnephrectomy, on the other hand, remained well within the normotensive range, with many values at or below the low limit of the normal. This is in dramatic contrast to the data from rats with intact parathyroid glands, as may readily be seen when the curves of mean values from FIGURES 24 and 25 are overlapped (FIGURE 26). Thus, it would seem that the early phase of hypertension following removal of the kidneys is dependent upon the presence of the parathyroid glands.

It is of interest that parathyroidectomized rats survived removal of the kidneys somewhat longer than those with intact parathyroid glands, as will be demonstrated again later, although in both groups the reniprival life span was reduced as a result of the added strain entailed in the repeated immobilization for the blood pressure measurements.

Pathologic-Anatomical Changes

The sites of the most frequent and severe injury following bilateral nephrectomy were the heart, the arterial tree, and the gastrointestinal tract. The gross and microscopic appearance of the lesions were indistinguishable from the picture of early lesions following renal injury and described in detail in the first part of this paper, except that muscular necrosis at all sites developed earlier in nephrectomized rats. In fact, under certain experimental conditions (injection of parathyroid extract), advanced pathologic-anatomical alterations, produced by renal injury only after a minimum interval of 5 to 7 days, were condensed into a span of less than 24 hours. On the other hand, because of the short life span of untreated reniprival rats, these changes obviously never reached the later and particularly the healing stages of renogenic injury.

Hormonal Mechanism and Interrelations

Nephrectomy and parathyroid extract. The development of cardiovascular and smooth muscle necrosis as a consequence of bilateral nephrectomy is exemplified with the group of 20 male rats shown in TABLE 7. Rats Nos. 1 to 10 (Group A) were nephrectomized only, whereas rats Nos. 11 to 20 (Group B)

TABLE 7

INFLUENCE OF PARATHYROID HORMONE UPON DEVELOPMENT OF CARDIOVASCULAR AND SMOOTH MUSCLE NECROSIS IN THE NEPHRECTOMIZED ALBINO RAT

(Male Animals 4 to 6 Months of Age)

Group	Description of procedures	Rat No.	Survival post-nephrectomy (days)	Macroscopic pathology*		
				Necrotizing myocarditis	Necrosis of aortic media	Necrosis of gastric muscularis
A	Nephrectomy only	1	3	−	−	−
		2	2	−	−	−
		3	4	−	+	−
		4	3	++++	++	−
		5	3	+	+	−
		6	3	+	−	−
		7	3	++	+	−
		8	3	+	+	−
		9	3	−	++	−
		10	3½	+	−	−
B	Nephrectomy plus parathyroid ext., 100 I.U. on first and second days postnephrectomy	11	3	+++	+++	−
		12	3	++++	+	−
		13	3	++++	+++	−
		14	3	++++	+++	−
		15	3½	++++	++	++
		16	3	+	++++	++
		17	3	+	++++	−
		18	3	++	++++	++
		19	3	+++	+++	−
		20	3	+++	+++	−

Classification of lesions: − none; + minimal, localized; ++ moderate and more extensive; +++ severe and widespread; and ++++ maximal degree.

received 100 units of parathyroid extract on the first as well as on the second day postnephrectomy.

It is apparent that, except for rat No. 2, all animals lived 3 days or longer, and there was no remarkable difference in survival time between the animals of Groups A and B. On the other hand, there was a striking difference in incidence and, particularly, the degree of grossly visible myocardial, aortic, and gastric injury.

In the "control" nephrectomy group (Rats Nos. 1 to 10), 8 rats had lesions in either heart or aorta or both, but in only one animal (No. 4) was the injury severe, and in only 2 additional animals (Nos. 7 and 9) were the changes moderately extensive in character. No grossly visible damage was encountered in the stomach of any animal from this group.

Administration of a total of 200 I.U. of parathyroid extract brought about a most dramatic and rather uniform aggravation of the cardiovascular and gastric injury, which under these conditions was severe in every single animal in either heart or aorta and usually in both of these organs. In addition, 3 animals of Group B manifested necrosis in the muscularis of the stomach.

Nephrectomy and parathyroidectomy. It was reasoned that, if the parathyroid hormone is prominently involved in the mechanism of muscular necrosis,

TABLE 8

INFLUENCE OF PARATHYROID HORMONE UPON DEVELOPMENT OF CARDIOVASCULAR AND SMOOTH MUSCLE NECROSIS IN THE NEPHRECTOMIZED ALBINO RAT

(Male animals 4 to 6 Months of Age)

Group	Description of procedures	Rat No.	Survival post-nephrectomy (days)	Macroscopic pathology*		
				Necrotizing myocarditis	Necrosis of aortic media	Necrosis of gastric muscularis
C	Parathyroidectomy (Px) 10 days prior to nephrectomy (Nx)	21	3	−	−	−
		22	3	−	−	−
		23	4	−	−	−
		24	4	−	−	−
		25	5	−	−	−
		26	3¾	−	−	−
		27	4¼	−	−	−
		28	4¼	−	−	−
		29	5	−	−	−
		30	†			
D	Px 10 days prior to Nx; parathyroid ext., 100 I.U. on first and second days post Nx	31	2¾	−	−	−
		32	2¾	+++	++++	++
		33	2½	−	−	++
		34	2½	++	++++	++
		35	2¾	+	−	−
		36	2½	++++	++	++
		37	2½	−	−	−
		38	2¾	−	−	−
		39	2½	+	+	−
		40	2½	+	−	−

* For classification of lesions see TABLE 7.

† Died before nephrectomy.

removal of the parathyroid glands prior to nephrectomy might prevent the emergence of cardiovascular necrosis; this was indeed the case. TABLE 8 illustrates findings in 2 groups of 10 male rats each, all of which had been parathyroidectomized 10 days prior to nephrectomy. In the upper half of the table it may be seen that, in the absence of the parathyroid glands, cardiovascular and gastric necrosis does not occur when the kidneys are removed, although the animals actually survive for a distinctly longer period than those shown in TABLE 7. In fact, 6 of the 9 rats in Group C survived at least 4 days, and 2 of these lived 5 days, and yet none of the animals in Group C showed even the mildest form of the characteristic lesions.

On the other hand, when these conditions were modified merely by the administration of parathyroid extract on the first and second days postnephrectomy (as shown in the lower half of TABLE 8), typical alterations in heart, aorta, and stomach were readily induced. This is the more significant, since survival time was uniformly shortened by the hormone injections to a maximum span of less than 3 days. Six of 10 animals in Group D manifested cardiovascular injury, and in 3 of these (Nos. 32, 34, and 36), the injury was severe in at least 1 of the 2 organs. In addition, these 3 animals and rat No. 33 also developed necrosis of the gastric muscularis.

These data, therefore, are entirely in line with our findings using the method of standard renal injury; namely, in that the parathyroid hormone is responsible for the development of muscular necrosis even though the ultimate mechanism is as yet unexplained.

Further studies revealed that parathyroid extract in a dosage adequate to produce severe degrees of cardiovascular necrosis in parathyroprival, reniprival rats (100 to 200 units daily) was unable to elicit hypertension in such animals. Similarly, these same amounts of exogenous hormone produced no noticeable accentuation of the hypertensive phase when given to reniprival rats with intact parathyroid glands.

It thus appears that commercially available parathyroid extract cannot substitute for the rat's own parathyroid hormone in the mechanism of early reniprival hypertension. The development of cardiovascular and smooth muscle necrosis of maximum severity in the complete absence of hypertension emphasizes again that hypertension is not required for the development of cardiovascular necrosis. It suggests further that the mechanism responsible for the production of muscular necrosis is not identical with the one involved in early reniprival hypertension.

Nephrectomy and adrenal cortical hormones. In previous studies using standard renal injury we reasoned that excess production of parathyroid hormone is mediated through the adrenal cortex, and that mineralocorticoids are of prime importance in the direct or indirect activation of the parathyroid glands, whereas glucocorticoids were essential for survival, but had no such effect.[12] It appeared of particular interest, therefore, to re-examine this differential action of mineralocorticoids and glucocorticoids in the absence of the kidneys.

Accordingly, 2 subgroups of 5 rats each were pretreated with cortisone and DCA, respectively, nephrectomized, and continued on these hormones until death. The results are illustrated in TABLE 9.

TABLE 9

INFLUENCE OF DCA OR CORTISONE UPON THE DEVELOPMENT OF CARDIOVASCULAR AND SMOOTH MUSCLE NECROSIS IN THE NEPHRECTOMIZED ALBINO RAT

(Male Animals 4 to 6 Months of Age)

Group	Description of procedures	Rat No.	Survival post-nephrectomy (days)	Macroscopic pathology*		
				Necrotizing myocarditis	Necrosis of aortic media	Necrosis of gastric muscularis
E	DCA, 3 mg. daily, starting 2 days prior to nephrectomy	41	3½	++	++++	++
		42	5½	++++	++++	++++
		43	4	++	+++	++
		44	4	++++	++++	+++
		45	4	++	++++	+++
F	Cortisone, 3 mg. daily, starting 2 days prior to nephrectomy	46	4½	+++	++++	+++
		47	3½	−	−	−
		48	3½	+	++	−
		49	3½	−	+	−
		50	3½	−	++	−

* For classification of lesions see TABLE 7.

It is apparent that all 5 DCA-treated rats (Group E) showed severest degrees of necrosis in the aorta and moderate to severe degrees in heart and stomach. The adrenals of these animals were greatly enlarged, ranging from 94 to 126 mg. moist weight.

In the cortisone group (F), on the other hand, only one rat (No. 46) showed the same degree of severe injury, one was completely negative (No. 47) and, in the remaining 3 animals, there were 1 minimal and 3 moderate lesions in the aorta and 1 minimal lesion in the myocardium. The adrenal weights suggested a possible correlation, since No. 46 exhibited by far the greatest increase (100 mg. in a rat weighing 345 gm.), whereas No. 47 had the smallest adrenals (66 mg. in a rat weighing 398 gm.).

It is of particular interest that the DCA-treated rats appeared stronger and more alert after nephrectomy than did their cortisone-treated counterparts. In fact, each animal of the latter group was very sick and weak during its entire reniprival life span, which was somewhat shorter than that of the DCA-treated rats. This is a complete reversal of our previous observations with standard renal injury.

If one compares these results with the findings in the nephrectomized rats with and without the administration of parathyroid hormone, as shown in TABLE 7, it would appear that, in the dosage employed here, excess of DCA provides the type of maximal aggravation observed with parathyroid extract administration, whereas cortisone in the same dosage neither prevents nor aggravates the alterations induced by nephrectomy alone.

Nephrectomy-adrenalectomy and adrenal cortical hormones. It was realized that a clear-cut answer on the differential effect of glucocorticoids and mineralocorticoids could be obtained only in the absence of the animal's own adrenal

glands. The experiment depicted in TABLE 9 was therefore repeated in identical fashion, except that this time the adrenals were removed simultaneously with the kidneys (Groups H and I of TABLE 10). Moreover, 2 additional groups of rats were employed. One served as a control group (G) of nephrectomized and adrenalectomized rats without hormone administration, and the second group (J) received both cortisone and DCA.

It is apparent that the life span of nephrectomized rats is definitely shortened in the absence of the adrenals, and that cardiovascular and smooth muscle necrosis fails to develop under these conditions (Group G). DCA in the same dosage as employed in the preceding experiment causes a substantial increase in the survival time (Group H). In addition, 4 of the 5 DCA-treated rats show aortic lesions, and 3 of these also have gastric necrosis. However, the changes are far less severe than in the presence of the adrenals. In line with the findings in TABLE 9, the cortisone group (I) of TABLE 10 again resembles the control group. The combination of DCA and cortisone (Group J), on the other hand, permitted the emergence of severe damage in the aorta and moderate to severe damage in the stomach in 4 of 5 rats. In addition, this group

TABLE 10

INFLUENCE OF DCA AND/OR CORTISONE UPON THE DEVELOPMENT OF CARDIOVASCULAR AND SMOOTH MUSCLE NECROSIS IN THE NEPHRECTOMIZED-ADRENALECTOMIZED RAT

(Male Animals 4 to 6 Months of Age)

Group	Description of procedures	Rat No.	Survival post-nephrectomy (days)	Macroscopic pathology*		
				Necrotizing myocarditis	Necrosis of aortic media	Necrosis of gastric muscularis
G	Nephrectomy-adrenalectomy (Nx-Ax) only	51	2¼	−	−	−
		52	2¼	−	−	−
		53	2	−	−	−
		54	2½	−	−	−
		55	2¾	−	−	−
		56	1½	−	−	−
H	DCA, 3 mg. daily, starting 2 days prior to Nx-Ax	57	5	−	++	++
		58	4	−	++	++
		59	3½	−	−	−
		60	4½	−	++	++
		61	2½	−	+	−
I	Cortisone, 3 mg. daily, starting 2 days prior to Nx-Ax	62	2	−	−	−
		63	2	−	−	−
		64	3	−	−	−
		65	3	−	−	−
		66	2½	−	−	−
J	DCA + cortisone, 3 mg. each daily, starting 2 days prior to Nx-Ax	67	3	−	−	++
		68	4	+++	+++	−
		69	4	−	++++	+++
		70	4	+	++++	++
		71	3½	++++	++++	+++

* For classification of lesions see TABLE 7.

was the only one among those depicted in TABLE 10 in which necrotizing myocarditis was encountered (animals Nos. 68, 70, and 71). It will be noted further that the life span is again substantially prolonged, as was seen with the administration of DCA alone (Group H).

The pronounced reduction in incidence and severity of reniprival muscular necrosis, when the identical dosages of either DCA or cortisone were administered to adrenalectomized rats, serves to emphasize the importance of the animals' own adrenal cortical hormones. It further suggested that "whole adrenal cortex" may have a potentiating effect upon the muscular injury, a possibility that has received additional support from the amazing increase in severity of lesions under the influence of the simultaneous administration of glucocorticoid and mineralocorticoid (Group J).

In order to distinguish between these two possibilities, a true synergistic effect of the hormone combination or a simple intensification based on increased dosage of either or both glucocorticoid and mineralocorticoid, the experiment was repeated exactly as illustrated here, except that the dosage of adrenocortical hormones was raised from 3 to 10 mg. daily. No additional control group was included in this study. The results are illustrated in TABLE 11.

It is apparent that under the increased hormone dosage severe aortic lesions were induced in each of the three experimental groups; that is, under the influence of either DCA (Group K), or cortisone (Group L), or a combination of

TABLE 11

INFLUENCE OF DCA AND/OR CORTISONE UPON THE DEVELOPMENT OF CARDIOVASCULAR AND SMOOTH MUSCLE NECROSIS IN THE NEPHRECTOMIZED-ADRENALECTOMIZED RAT

(Male Animals 4 to 6 Months of Age)

Group	Description of procedures (nephrectomy—Nx; adrenalectomy—Ax)	Rat No.	Survival post-nephrectomy (days)	Macroscopic pathology*		
				Necrotizing myocarditis	Necrosis of aortic media	Necrosis of gastric muscularis
K	DCA, 10 mg. daily, starting 2 days prior to Nx-Ax	72	4½	++	++++	++
		73	3¾	++	++++	−
		74	3½	+++	++++	+++
		75	<4	+	++++	++
		76	4½	++	++++	+++
L	Cortisone, 10 mg. daily, starting 2 days prior to Nx-Ax	77	2½	++	++++	−
		78	2½	−	++++	−
		79	1¾	−	+	++
		80	2½	++	++++	++
		81	2½	+	−	−
M	DCA + cortisone, 10 mg. each daily, starting 2 days prior to Nx-Ax	82	2½	−	++++	−
		83	2½	+++	++++	−
		84	2½	+++	++++	++
		85	2¼	−	+	+
		86	2¾	+++	++++	−

* For classification of lesions see TABLE 7.

both hormones (Group M). The greater degree of necrosis in the muscularis of the stomach observed in Group K can be accounted for on the basis of the substantial differences in survival time since, as a rule, a life span of 3½ to 4 days is required for the development of severe lesions at this site (see also TABLE 9, Groups E and F, and TABLE 10, Group J). As in the 2 preceding experiments, survival time is again lengthened in the DCA-treated animals, whereas the larger cortisone dosage causes no improvement in survival time (compare with Groups G and I of TABLE 10) and, when combined with DCA, actually cancels out the lengthening of survival time produced by the mineralocorticoid alone. Again, the 5 rats receiving DCA only appeared definitely more vigorous and alert than the 10 cortisone-treated animals (FIGURE 27).

This is in striking contrast to our observations with standard renal injury. It was found then that DCA could not extend the survival time of adrenalectomized rats for more than 24 hours following renal block, whereas cortisone was highly efficient in this respect.[23] It is of particular interest that, despite the short life span of less than 3 days in both cortisone-treated groups, cardiovascular necrosis of such severity could be produced. Since untreated nephrectomized and adrenalectomized rats survive about as long (Group G, TABLE 10), the lack of cardiovascular necrosis in the complete absence of adrenal cortical hormones therefore could not be explained on the basis of the short life span. Additional evidence for this viewpoint is contained in subsequent studies.

Combined nephrectomy-adrenalectomy in parathyroprival animals. It was shown in parathyroprival rats that the administration of parathyroid extract

FIGURE 27. Appearance of parathyroprival albino rats on the third day following bilateral adrenalectomy-nephrectomy. The normal-appearing rat on the right was treated with DCA; the flabby, atonic, and completely immobile rat on the left received cortisone instead.

elicited typical muscular necrosis at the predilection sites, but it had yet to be demonstrated whether this injury would occur in the complete absence of adrenal cortical hormones.

Attempts to investigate this point in our previous studies with "renal block" were not successful because of the poor survival of adrenalectomized rats following standard renal injury. It was reasoned that, if the primary function of adrenal cortical hormones in the mechanism of muscular necrosis was the stimulation of the parathyroid gland to release of excess hormone, then administration of parathyroid extract to parathyroprival rats in amounts known to be adequate for the production of muscular necrosis should induce the typical injury in the complete absence of the adrenals, provided survival of such animals was of sufficient duration. Moreover, no pronounced aggravation of the injury should be expected under these experimental conditions if adrenal corticoids were supplied concomitantly with parathyroid extract since, in the absence of the parathyroid gland, the level of parathyroid hormone could not be enhanced through the administration of adrenal corticoids. An investigation of this problem is depicted in TABLE 12.

In this experiment three groups of parathyroidectomized rats were subjected to simultaneous bilateral nephrectomy-adrenalectomy and administration of the following hormones.

Group N (rats Nos. 87 to 96) received parathyroid extract only, in amounts known to induce substantial cardiovascular necrosis in parathyroidectomized rats following nephrectomy (TABLE 8, Group D).

Group O (rats Nos. 97 to 106) received excessive amounts of adrenal corticoids only. The dosage of 10 mg. each of DCA and cortisone daily was shown to insure the development of cardiovascular necrosis of maximal severity within 2½ days following nephrectomy-adrenalectomy in rats possessing intact parathyroid glands (TABLE 11, Group M).

Group P (rats Nos. 107 to 116) received both parathyroid extract and adrenal corticoids in the same dosages as used in groups N and O.

It can be seen that in Group N survival time is rather short (1 or 2 days) and definitely shorter than in the presence of the adrenal gland (TABLE 8, Group D), and yet incidence and severity of lesions are apparently not substantially different from those encountered in the presence of the adrenal gland. In fact, severe muscular necrosis was observed in rats surviving nephrectomy-adrenalectomy for only 24 to 36 hours (rats Nos. 94 and 95). These findings indicate that adrenal hormones are not essential in the mechanism of muscular necrosis induced by parathyroid extract and that no direct peripheral synergistic effect appears to exist between parathyroid extract and adrenal corticoids.

This is further emphasized by the finding in Group O, where excessive dosages of DCA and cortisone in combination failed to produce any lesions in the absence of the parathyroid glands despite the fact that the life span was considerably extended by their administration.

Combined administration of adrenal corticoids and parathyroid extract (Group P) resulted in extension of survival time similar to that seen in Group

TABLE 12

INFLUENCE OF PARATHYROID AND ADRENAL CORTICAL HORMONES UPON THE DEVELOPMENT OF CARDIOVASCULAR NECROSIS IN THE PARATHYROIDECTOMIZED, NEPHRECTOMIZED, AND ADRENALECTOMIZED RAT

(Male and Female Animals 4 to 6 Months of Age)

Group	Description of procedures (parathyroidectomy—Px; nephrectomy—Nx; adrenalectomy—Ax)	Rat No.	Survival post-nephrectomy (days)	Macroscopic pathology*		
				Necrotizing myocarditis	Necrosis of aortic media	Necrosis of gastric muscularis
N	Px, parathyroid, 100 I.U. daily, post Nx-Ax	87	1	−	−	−
		88	2	++	−	−
		89	2	++	+	−
		90	>2	++	++	++
		91	>2	+++	+	++
		92	0.¾	−	−	−
		93	1	++	+	−
		94	1	+++	−	−
		95	1½	++++	++	+++
		96	1½	−	−	−
O	Px, DCA, and cortisone, 10 mg. each daily, starting 2 days prior to Nx-Ax	97	3½	−	−	−
		98	3½	−	−	−
		99	2½	−	−	−
		100	<4	−	−	−
		101	<4	−	−	−
		102	3½	−	−	−
		103	3	−	−	−
		104	3	−	−	−
		105	2	−	−	−
		106	3½	−	−	−
P	Px, DCA, and cortisone, 10 mg. each daily, starting 2 days prior to Nx-Ax; parathyroid, 100 I.U. daily, post Nx-Ax	107	3	++	+++	−
		108	3	+	−	−
		109	3	−	−	−
		110	3	+++	+++	−
		111	3	+++	+++	−
		112	2½	−	−	+
		113	3¼	+++	+++	++
		114	3½	−	++	++
		115	>4	−	+++	+++
		116	2¼	−	−	+

* For classification of lesions see TABLE 7.

O, and yet there was a definite increase solely in the severity of aortic damage and not in over-all incidence of muscular injury, if compared with Group N. It should be remembered in this connection that the rats of Group P received actually two or three times as much parathyroid extract as the animals of Group N because they survived longer. Moreover, in the presence of the animal's own parathyroid gland and without additional parathyroid extract this same dosage of adrenal corticoids was responsible for the emergence of lesions of maximal severity in the heart and in the aorta (TABLE 11, Group M).

The evidence appears to support the conclusion that adrenal corticoids do not aggravate muscular necrosis induced by parathyroid hormone by a direct peripheral synergistic action.

Since parathyroid extract induced typical muscular necrosis in the absence of the adrenals, whereas adrenal corticoids in excessive dosage were unable to evoke even minimal muscular necrosis in the absence of the parathyroid gland, it must be assumed that the function of adrenal corticoids in the mechanism of this experimental lesion is to permit or to trigger the release of excessive amounts of parathyroid hormone, which in turn initiate the development of disseminated muscular necrosis.

Importance of the Adrenal Cortex-Parathyroid "Axis" in the Mechanism of Reniprival Cardiovascular Necrosis

Since the data on nephrectomized rats presented above strongly suggested the existence of an adrenal cortex-parathyroid axis, it appeared of particular interest to investigate a possible "trophic" effect of the adrenal cortex upon the parathyroid gland, somewhat in the nature of the trophic effects of the anterior pituitary upon other endocrine glands. To that end, a number of experiments were repeated in a manner closely similar to some of those described under simultaneous adrenalectomy-nephrectomy (TABLES 10 and 11) except that the rats were adrenalectomized 10 days in advance of nephrectomy and maintained in the interval on either saline or various dosages of adrenal corticoids.

Experiments of this type are illustrated in the next three tables. TABLE 13 shows that adrenalectomized rats (Group 1) drinking saline survived nephrectomy about as well as rats with intact adrenals, 3.2 days on the average (TABLE 7, Group A), but did not develop muscular necrosis.

Maintenance on 3 mg. DCA daily did not influence the reniprival life span, but allowed for the emergence of 2 mild and 1 moderate lesion (Group 2).

It was necessary to raise cortisone dosages from 3 to 10 mg. after 3 days, because without saline the animals did poorly even before nephrectomy; in fact, one animal died before reaching this stage. Survival after nephrectomy was extremely poor, and only a single mild myocardial lesion was encountered among 4 animals (Group 3).

Combined administration of both hormones in 3-mg. dosages (Group 4) resulted in survival similar to that seen with DCA alone (Group 2), but the incidence and severity of lesions were again strikingly increased (compare with TABLE 10, Group J), apparently as with simultaneous adrenalectomy-nephrectomy, on the basis of higher dosage of adrenal corticoids.

It is of interest that all data depicted in TABLE 13, resemble very closely those obtained with simultaneous removal of adrenals and kidneys (TABLE 10).

In the former study, it will be recalled, raising the dosage of either DCA or cortisone produced a high incidence of muscular necrosis (TABLE 11, Groups K and L). However, when a similar maneuver was tried in adrenoprival rats (that is, maintenance on 10 mg. of either DCA or cortisone, beginning with the day of adrenalectomy) the results were somewhat different with regard to the cortisone group (TABLE 14).

In this larger dosage DCA is responsible for excellent survival (Group 5). Actually these rats would have lived for more than 4 days. They were in good

TABLE 13

ADRENAL CORTICOIDS AND THE DEVELOPMENT OF MUSCULAR NECROSIS IN ADRENALECTOMIZED (Ax) RATS FOLLOWING NEPHRECTOMY (Nx)

(Ax 10 Days Prior to Nx; Male Animals 4 to 6 Months of Age)

Group	Description of procedures	Autopsy number	Survival post Nx (days)	Macroscopic pathology*		
				Necrotizing myocarditis	Necrosis of aortic media	Necrosis of gastric muscularis
1	No hormones	2748	3¾	−	−	−
		2739	2	−	−	−
		2745	3½	−	−	−
		2744	3¾	−	−	−
		2743	3	−	−	−
2	DCA, 3 mg. daily, starting with Ax	2821	3¼	−	−	−
		2822	3¾	−	−	−
		2823	3½	−	++	−
		2827	3¾	+	−	−
		2828	4¼	−	+	−
3	Cortisone, 3 mg. to 10 mg. daily, starting with Ax	2817	1¾	−	−	−
		2818	1¾	+	−	−
		2816	1¼	−	−	−
		2819	2	−	−	−
4	DCA and cortisone, 3 mg. each daily, starting with Ax	2829	4¼	+	++++	+++
		2830	4¼	++	++++	++++
		2820	3	+++	+	++
		2826	3½	+++	+++	++
		2824	3½	+	++++	−
		2825	3¾	++++	++	−

* For classification of lesions see TABLE 7.

condition when it was necessary to sacrifice them at the end of the fourth day, since this was the maximum possible span of continuous observation in this particular experiment. It will be noted that the incidence and severity of organic lesions are about equal to those observed with the DCA-cortisone combination of 3 mg. each (TABLE 13, Group 4).

In sharp contrast, cortisone in identical dosage (Group 6) is definitely toxic, reducing the life span for untreated rats from more than 3 days to 2 days. This shortening of survival might be responsible, in part, for the lack of aortic and gastric lesions. A more plausible explanation, however, seems to lie in a pronounced diminution of the responsiveness of the parathyroid gland to nephrectomy when the adequate supply of mineralocorticoids had been interrupted for a considerable length of time before removal of the kidneys. This would serve to explain the complete absence of aortic injury in Group 6 and its very conspicuous presence in the rather similar Group L of TABLE 11, where the adrenals were removed at the time of nephrectomy only. Hence, a special "trophic" effect of DCA (Group 5 of TABLE 14) or of the endogenous mineralocorticoid (Group L of TABLE 11) upon the parathyroid gland cannot be dismissed as a possibility.

TABLE 14

ADRENAL CORTICOIDS AND THE DEVELOPMENT OF MUSCULAR NECROSIS IN ADRENALECTOMIZED (Ax) RATS FOLLOWING NEPHRECTOMY (Nx)

Group	Description of procedures	Autopsy number	Survival post Nx (days)	Macroscopic pathology*		
				Necrotizing myocarditis	Necrosis of aortic media	Necrosis of gastric muscularis
5	DCA, 10 mg. daily, starting with Ax	2931	4	++	+++	++++
		2932	4	+++	++++	++
		2933	4	+	++++	+++
		2934	4	−	+	++
		2935	4	+++	+++	++++
6	Cortisone, 10 mg. daily, starting with Ax	2920	2	+	−	−
		2921	2	+++	−	−
		2922	2	−	−	−
		2923	2	+	−	−
		2924	2	−	−	−
		2925	2	−	−	−

* For classification of lesions see TABLE 7.

This experiment indicates that in adrenalectomized rats the parathyroid glands can respond to nephrectomy with excess production of hormone only under maintenance with large doses of adrenal corticoids. It seemed to follow that this same dosage of adrenal corticoids might be less effective if administration were delayed for a considerable time after adrenalectomy. This was indeed the case.

When the experiment with the 2 groups showing a high incidence of muscular necrosis (Group 4 of TABLE 13 and Group 5 of TABLE 14) was repeated exactly as done before, except that after adrenalectomy the rats were maintained on saline for 8 days before being put on adrenal corticoids, there was a pronounced reduction in the incidence and severity of lesions without any substantial alteration of the reniprival life span (TABLE 15, groups 7 and 8).

It should be remembered that this 2-day priming with the same dose of adrenal corticoids, as done in the present experiment, produced a high incidence of severe cardiovascular lesions when the adrenals were removed at the time of nephrectomy only (TABLE 10, Group J, and TABLE 11, Group K). This fact suggests that the responsiveness of the parathyroid gland decreases in the absence of adrenal cortical hormones.

Résumé of Results

If the main findings of the nephrectomy studies as outlined in some detail in TABLES 7 to 15 are condensed into a single simplified table that also includes additional confirmatory experiments, the essential results can be brought into sharper focus. For the sake of greater clarity and conciseness, the data of groups exposed to similar and comparable experimental conditions were pooled, and cardiovascular necrosis was considered a single entity representa-

TABLE 15

ADRENAL CORTICOIDS AND THE DEVELOPMENT OF MUSCULAR NECROSIS IN ADRENALECTOMIZED (Ax) RATS FOLLOWING NEPHRECTOMY (Nx)

(Ax 10 Days Prior to Nx; Male Animals 4 to 6 Months of Age)

Group	Description of procedures	Autopsy number	Survival post Nx (days)	Macroscopic pathology*		
				Necrotizing myocarditis	Necrosis of aortic media	Necrosis of gastric muscularis
7	DCA, 10 mg. daily, starting 2 days prior to Nx	2794	4¼	−	+	+
		2787	3¾	−	+	++
		2790	4	−	+++	+++
		2788	3¾	−	−	+
		2791	3½	−	−	−
8	DCA and cortisone, 3 mg. each daily, starting 2 days prior to Nx	2926	3	−	−	−
		2927	3	−	+++	−
		2928	3	++	−	−
		2929	3	+	++	−
		2930	3	++++	+++	−

* For classification of lesions see TABLE 7.

tive of the typical injury at all predilection sites. This résumé of results is presented in TABLE 16, which illustrates the following facts:

Albino rats survive bilateral nephrectomy without treatment for about 3½ days on the average, and manifest a high incidence of predominantly moderate cardiovascular necrosis (Group 1). There are 12 moderate and 5 severe lesions among 20 rats, accounting for a total incidence of 85 per cent.

Parathyroid extract injected postnephrectomy causes a most dramatic increase in the severity of cardiovascular necrosis (Group 2) despite a slight reduction of the life span. There are 22 severe and 5 moderate lesions among 28 rats; hence the total incidence is 97 per cent.

Removal of the parathyroid gland prior to nephrectomy completely prevents the emergence of cardiovascular necrosis despite an actual lengthening of the mean survival time from 3.5 to 4 days (Group 3).

Substitution of parathyroprival-reniprival rats (as shown under Group 3) with parathyroid extract in amounts that must be considered moderate for the rat causes a high incidence of typical cardiovascular injury (Group 4) despite the fact that the life span is substantially reduced. There are 5 moderate and 4 severe lesions (total incidence 60 per cent).

If the adrenals are removed simultaneously with the kidneys (Group 5) the life span is shortened considerably (compare with Group 1). In addition, no muscular necrosis was found in any of these rats.

In such animals treatment with DCA causes a pronounced increase in survival time (4 days) and a high incidence of cardiovascular necrosis (Group 6). There are 8 moderate and 9 severe lesions among 20 rats, making a total incidence of 85 per cent.

Under the same conditions cortisone in equal dosage may shorten the life

TABLE 16
PATHOGENESIS OF CARDIOVASCULAR NECROSIS INDUCED BY NEPHRECTOMY IN THE ALBINO RAT*

Group	Organs excised (x); kidneys—N; parathyroids—P; adrenals—A	No. of rats	Hormone administered	Approx. mean survival (days)	Cardiovascular lesions		
					Number		Total incidence (per cent)
					Moderate + ++	Severe +++ ++++	
1	Nx	20	—	3½	12	5	85
2	Nx	28	Parathyroid	3¼	5	22	97
3	Px Nx	20	—	4	—	—	0
4	Px Nx	15	Parathyroid	2½	5	4	60
5	Ax Nx	22	—	2½	—	—	0
6	Ax Nx	20	DCA	4	8	9	85
7	Ax Nx	20	Cortisone	2¼	5	4	45
8	Ax Nx	16	DCA and cortisone	3¼	1	14	93
9	Px Ax Nx	10	Parathyroid	1½	4	3	70
10	Px Ax Nx	14	DCA and cortisone	3¼	—	—	0
11	Px Ax Nx	10	Parathyroid, DCA and cortisone	3	2	5	70

* Condensation of TABLES 7 to 15, with additions.

span (Group 7), yet it likewise enables the emergence of typical alterations, although in a smaller percentage of cases, the total incidence being 45 per cent.

If cortisone and DCA are given simultaneously (Group 8) the life span is found to lie between that of rats receiving either of these corticosteroids separately. The incidence of severe changes, on the other hand, is markedly increased and, actually, about the sum of that of the separate hormones.

If the adrenals are removed simultaneously with the kidneys in parathyroprival rats and such animals are given 100 units of parathyroid extract daily thereafter, survival is extremely poor (only 1½ days on the average), yet a high incidence of necrosis is found at the predilection sites (Group 9). Hence the presence of adrenal corticoids is not essential for the production of these lesions.

Moreover, cortisone and DCA administered instead of parathyroid extract in excessive dosages under these same experimental conditions fail to invoke even minimal cardiovascular alterations despite the fact that by their presence survival time is lengthened by almost 2 days (Group 10).

Simultaneous administration of adrenal corticoids and parathyroid extract in the dosages used in Group 9 and 10 did not result in any substantial aggravation of the pathologic-anatomical picture (Group 11) and certainly not beyond that expected on the basis of parathyroid dosage alone.

The data of groups 9 and 11 appear to exclude a peripheral synergistic action of adrenal corticoids and parathyroid hormone. This view receives additional support from the closely similar findings of Group 4, which also contains parathyroprival rats receiving parathyroid, but whose adrenals were left intact. It is quite obvious that in this situation exogenous parathyroid hor-

mone was not more effective in inducing muscular necrosis than it was in the complete absence of the adrenals (Group 9).

All these observations are fully in accord with the concept of an adrenal cortex-parathyroid axis in the mechanism of reniprival cardiovascular necrosis, since the parathyroid factor involved in the production of muscular necrosis can apparently be manufactured or released in sufficient excess only in the presence of adequate amounts of adrenal corticoids.

Discussion

On the basis of the studies presented it is concluded that disseminated cardiovascular and smooth muscle necrosis, occurring in the rat either as a consequence of pronounced impairment of renal excretory function or following bilateral nephrectomy, is elicited by the same mechanism, that is, by overproduction of parathyroid hormone. In both instances the development of muscular necrosis is readily prevented by removal of the parathyroid glands. Also, in both instances, adrenal cortical hormones appear of importance as direct or indirect mediators of parathyroid hyperfunction. Thus, in parathyroprival rats adrenal corticoids were unable to induce the typical pathologic-anatomical alterations and also did not enhance muscular necrosis produced by exogenous parathyroid hormone. The powerful potentiating effect of adrenal corticoids upon muscular necrosis in the presence of the parathyroid glands is therefore consistent with the concept of an adrenal cortex-parathyroid "axis." In other words, adrenal cortical hormones are necessary to permit or to trigger the release of excess hormone from the parathyroid glands. The basic stimulus for the hormone release is supplied by severe impairment or absence of excretory renal function resulting in phosphate retention and other metabolic disturbances.

With regard to so-called renogenic muscular necrosis, the evidence derived from studies with reniprival rats eliminates the necessity of postulating the deleterious contribution of "toxins" manufactured by the damaged kidney. It also militates strongly against a detoxifying mechanism contained in the healthy kidney or a protective factor elaborated by this organ, since full protection against muscular damage can be achieved in the complete absence of the kidneys, namely, by prior parathyroidectomy.

The prodigious literature on renal and reniprival cardiovascular disease and hypertension contains many theories on renal factors and on extrarenal mechanisms believed to be involved in the development or prevention of these conditions.[27] However, while the role of the kidney and of the adrenal cortex and their relationships to disturbances of mineral metabolism, of fluid balance, and of hemodynamics as causative factors in cardiovascular disease have been studied exhaustively in the experimental animal and in man, surprisingly little attention has been paid to the possible significance of the parathyroid gland in the production of cardiovascular lesions.

Nevertheless it is commonly recognized that serious renal damage in the experimental animal as well as in human beings may result in secondary hyperparathyroidism with "metastatic calcification" in the cardiovascular system

and other tissue sites. In fact, the classic clinical entity is so well defined that it would seem to require little further elaboration here, except perhaps to emphasize that the existence of hyperparathyroidism may have remained unrecognized or may even have been mistakenly ruled out in many instances on the basis of normal blood calcium levels or the presence of anatomically normal parathyroid glands. Actually, neither of these observations should permit the exclusion of parathyroid hyperfunction since, in the subacute and chronic phases of experimental and clinical hyperparathyroidism, blood calcium levels may return to within the normal range and since, under physiological conditions, the parathyroid glands were shown to operate only at a small fraction (5 to 25 per cent) of their full capacity.[28] This wide margin of safety obviously allows for production and release of substantial excess of hormone without noticeable hypertrophy of the glandular parenchyma.[29] Moreover, it is conceivable that a specific vasoactive parathyroid factor, claimed to be separable from the calcium mobilizing activity of parathyroid extract,[5] is responsible for both spasm and necrosis of muscles.

In the experimental animal the deleterious effect of parathyroid hormone overdosage has been demonstrated by many workers.[30-34] McJunkin *et al.*[33] in particular have stressed that muscular necrosis invariably precedes calcification in the rat poisoned with parathyroid hormone. This viewpoint was later reiterated for similar lesions produced in the dog by Cantarow and his associates.[34]

A causative relationship between parathyroid hyperfunction and either experimental renal injury or bilateral nephrectomy has also been demonstrated. Pappenheimer,[35] for instance, has shown that albino rats after three-quarter nephrectomy and the development of progressive destructive renal lesions go on to develop secondary hyperparathyroidism in association with renal failure. In accord with these observations and in confirmation of our own findings,[10] Rather[36] has demonstrated the development of mediocalcinosis of the aorta in rats with advanced kidney disease following renal operations (three-quarter nephrectomy or unilateral nephrectomy and figure-eight ligature to the remaining kidney).

Ingalls and his co-workers[37] demonstrated that bilateral nephrectomy produced bone resorption in rats in 60 hours that was similar to the bone lesions produced in intact rats by injection of 400 units of parathyroid extract daily, the implication being that nephrectomy stimulates the parathyroid gland to hyperfunction. Stoerk[38] has reported that, in the absence of the kidneys, blood calcium is maintained at a normal level because of the increased activity of the intact parathyroids and that, after parathyroidectomy, calcium levels can be elevated by the injection of parathyroid hormone. He based his findings on serum calcium values obtained 48 hours postnephrectomy. Our own data accord with those of Stoerk, although we found substantial rises above the norm, particularly within the first 24 hours after removal of the kidneys.

Perusal of the literature indicates that in many instances in which Mönckeberg-type arteriosclerosis was produced in experimental animals, renal injury and secondary hyperparathyroidism might have been the underlying mechanism, although this possibility may have remained unrecognized.

Concerning the ultimate mechanism of muscular necrosis induced by excess parathyroid hormone, two possibilities must be considered: (1) parathyroid hormone contains an actual necrotizing factor, possibly unrelated to its calcium-mobilizing or phosphaturic activity; and (2) the hormone itself is not at fault, but mineral metabolic imbalances and possibly other disturbances created by its presence in excess are responsible for the muscle injury.

While a clear-cut answer to either possibility cannot be given at this time from data available in the literature, the second hypothesis appears far better supported. Many authors fail to mention muscular necrosis as a cardinal sign of parathyroid hormone poisoning in the apparent belief that extensive calcifications observed in the arterial tree and other tissue sites represent calcium salt deposits in healthy recipient tissue (true metastatic calcification). Others assume that tissue alterations are secondary to the encrustation with lime salts, a view probably based on findings in chronic hyperparathyroidism, where massive calcium deposits are surrounded by healthy tissue.

Undoubtedly, the derangement of the calcium and phosphate equilibrium in the blood and tissue of the body in the wake of renal failure constitutes at least an important contributory mechanism to the total picture of pathologic-anatomical alterations. With the retention of phosphates in partial renal insufficiency there is initially a concomitant decrease in the serum calcium level. Both the increased phosphate and the decreased calcium level in the blood stream may stimulate the parathyroid glands and induce mobilization of calcium salts from the bones. This results in elevation of the calcium level to near-normal or slightly above-normal levels. In view of the inadequate phosphate elimination, oversaturation of the blood with calcium and phosphate ions will promote the deposition of calcium phosphate into tissues where conditions are favorable.[39] It is conceivable that at these locations the early accumulation of calcium phosphate, although histologically not demonstrable, may participate in the production of cellular injury. In this connection it should be recalled that dehydrotachysterol[40] and the related vitamin D[41] in toxic dosage produce cardiovascular lesions similar to those described here.

In view of many similarities in the therapeutic and toxic actions of parathyroid hormone and vitamin D and its congeners, it has been suggested by a number of authors that vitamin D acts in part through the mediation of the parathyroid glands.[42-44] Other authors have challenged this view[45-51] and have proposed that the action of vitamin D is independent of the parathyroids.

Dikshit,[52] trying to find an answer to these divergent opinions, studied the effect of therapeutic and toxic dosages of vitamin D in thyroparathyroidectomized rats. He concluded that neither the physiological nor toxic action of vitamin D is mediated through the parathyroids. Unfortunately, he made no attempt to study chemical or pathologic-anatomical changes, but used a decrease in body weight and in food consumption to differentiate effects of hypervitaminosis D upon intact and thyroparathyroidectomized rats. Obviously, evaluation based on so unspecific a reaction does not allow for an unequivocal decision for or against a connection of vitamin D to the parathyroid gland. Moreover, this investigator's conclusions are at variance with the observation of Pappenheimer[53] that removal of the parathyroids makes it

difficult or impossible to induce rickets in rats. The classic studies of Albright and his associates,[54] on the other hand, appear to have shown convincingly that the therapeutic effects of vitamin D are similar to those of parathyroid hormone, yet independent of the parathyroid gland. However, the evidence is certainly less clear-cut in vitamin D or dehydrotachysterol poisoning, where early advanced impairment of renal function invariably dominates the picture and thus may be responsible for secondary hyperparathyroidism due to phosphate retention, as demonstrated in our studies with reniprival rats. The possible presence of secondary hyperparathyroidism in vitamin D poisoning can be inferred from the thorough work of Gillman and Gilbert,[55, 56] who showed that complete "thyroidectomy" in rats, which by necessity must have included the parathyroids, provided striking protection against vascular damage induced by excess of vitamin D.

The literature contains other similar examples of protection against cardiovascular damage by "thyroidectomy" when actually thyroparathyroidectomy was performed.

Salgado,[57] for instance, reported that "thyroidectomy" inhibited the emergence of myocarditis and nephrosclerosis produced in the unilaterally nephrectomized rat by DCA implantation and salt loading. Elsewhere in these pages he states that this protective effect was not abolished by thyroid administration. However, he failed to replace the missing parathyroid hormone.

A similar case of "mistaken identity" seems to be contained in the data of Roszkowski and Oester,[58] who reported complete protection of rabbits against development of epinephrine-induced aortic medionecrosis by "thyroidectomy." In his paper in this monograph Oester demonstrates that this protection was not abolished by administration of thyroxin or triiodothyronine. Since in both of these instances the parathyroids were removed with the thyroid, and since full thyroid replacement did not support the development of these lesions, it seems justifiable to ascribe the protective effect to removal of the parathyroids rather than of the thyroid gland, a possibility that was conceded by both Salgado and Oester.

The fact that excess thyroid hormone may aggravate many types of cardiovascular lesions is shown by Gillman and Gilbert[55] for vitamin D poisoning, by Oester and his associates[59] for arteriopathy induced by arterenol and epinephrine, and in our own studies for renogenic muscular necrosis. These facts should not automatically lead to the conclusion that thyroid removal will be protective, since complicated hormonal interrelations and mineral metabolic alterations may be involved rather than a direct effect of the thyroid hormone. This was shown to be the case in our studies, where thyroidectomy had no protective effect when the presence of excess parathyroid was assured. Moreover, the thyroidectomized dog has been shown to develop arterial necrosis and calcification under viosterol dosage more readily than the intact animal.[60-62]

The exact role of elevated serum calcium and phosphate levels or of increased ionized calcium in the development of cardiovascular necrosis is difficult to ascertain.

Ham[63] and Ham and Lewis[64] believe that in the rat, following a massive

parenteral dose of viosterol, calcification of the muscle fibers of the arterial media occurs without preceding anatomical change in these fibers, and that calcification is due simply to the inability of the serum to retain all the calcium, even in the vicinity of normal tissue. It is thus not dependent on degenerative changes in the recipient tissue. They believe that the same mechanism applies to calcification in poisoning with parathyroid hormone. Duguid,[65] on the basis of histological evidence, disagrees. He believes that the primary lesion in vitamin D poisoning consists in degeneration of the arterial smooth muscle, and that calcification follows this change. McAllister and Waters[62] concur with this viewpoint as regards the dog and emphasize that the arterial lesions are similar to those occurring in dogs with experimental renal disease.

The similarity between vitamin D and parathyroid hormone poisoning is indeed striking. Thus, Engel[66] has demonstrated that the parathyroid gland is implicated in maintaining the labile state of the organic matrix. Parathyroid extract administered to rats caused depolymerization and solution of the glycoprotein ground substance of bones and of the cartilage spiculae at the epiphysis. There is an associated rise in the serum mucoprotein level that appears to be related to the degree of bone change and, roughly, to the dose of parathyroid extract. Eisenstein and Graff,[67] using massive doses of vitamin D2 in rats, found increased seromucoid and calcium levels in the blood and accumulation of mucopolysaccharides at the particular locations in the heart, kidneys, and blood vessels where calcium deposition occurred. The pictures of the lesions shown are identical with those observed by us, including the myocarditis, which was unassociated with the deposition of calcium as assessed histologically.

In a thorough analysis of the mechanism of vitamin D injury Gillman and Gilbert,[56] on the other hand, conclude that hypercalcemia is not automatically accompanied by the deposition of calcium in the tissues, and that extensive calcification of the aorta and other organs can occur without any marked rise in the blood calcium. They believe that vitamin D induces two serial processes not necessarily dependent upon one another: namely, tissue damage followed by calcification. These two processes may be "uncoupled" so that tissue damage (that is, the necrotizing lesions of arteries and other injuries) may occur without calcification. Furthermore, Gillman and Gilbert believe that the arterial and cardiac lesions seen in vitamin D intoxication are dependent upon partial disorganization of the kidneys and that derangement of kidney function, in turn, is due to excess mobilization of calcium and phosphate by vitamin D. Finally, these investigators emphasize the fact that the arterial and myocardial lesions occurring in rats after excess vitamin D are reminiscent of those described in rats, rabbits, and dogs used for the investigation of hypertension by techniques aimed at severely disorganizing kidney function.[68-71]

The conclusions of Gillman and Gilbert[55, 56] on lesions seen in vitamin D poisoning have been cited at some length because they are fully in accord with the views held at this laboratory on cardiovascular injury observed in hyperparathyroidism. Like others before us,[33, 34] we believe that excess parathyroid hormone causes first muscular degeneration and necrosis and that deposition of calcium salts occurs in altered recipient tissue. The possible contribution

of substantially elevated calcium phosphate levels to the development of primary muscular injury is, of course, not excluded. Dissolved calcium salts are eluted during tissue fixation, and hence are not demonstrable histologically. They could, however, be pinpointed by chemical analysis of the tissues.

It should be added that parathyroid hormone poisoning probably underlies a variety of instances of extensive cardiovascular necrosis and calcification where renal injury is a consistent finding; this would therefore include vitamin D poisoning. However, it may entail in addition experimental and clinical disease which, at first glance, might seem to have little to do with the kidneys or the parathyroid glands.

The interesting work of Hartroft and his associates[72] on cardiovascular injury produced in the rat by choline deficiency represents a case in point. These workers described what they originally called "atheromatous" changes in the aorta and the carotid and coronary arteries of rats that in microscopic and histologic appearance were closely similar to the lesions that we obtained without choline deficiency and thus without "a state of chronic fat embolism" cited by these investigators as the reason for arterial medionecrosis. At this time the kidneys were not implicated, although every single animal that developed vascular lesions on choline-deficient diets showed pronounced renal impairment. Wilgram and Hartroft have since modified their stand[73] and have proposed that secondary hyperparathyroidism due to impairment of renal function may be as important as choline deficiency per se in the development of arterial lesions in the rat. Still later Wilgram and Blumenstein[74] reached the conclusion that the cardiovascular changes in choline deficiency are due primarily to the concomitant renal injury and can in fact be prevented by measures that lessen the embarrassment of the kidney (renal decapsulation). The apparent protection of rabbits against epinephrine-induced medionecrosis of the aorta by parathyroidectomy, inferred from the studies of Oester and his group[58] and mentioned earlier in this discussion, represent another likely example of unrecognized hyperparathyroidism.

The pathogenesis of arterial injury produced by epinephrine injections aroused great interest at the beginning of this century[75-77] as a possible cause of arteriosclerosis, an interest reawakened in more recent times to a large extent by the searching work and brilliant writing of Raab[78] on the pressor amines and their relationships to cardiovascular disease. Although the early studies cited and later confirmatory work[79] do not consider a renogenic origin of the arterial medionecrosis and calcification, there can be little doubt that repeated injections of excessive amounts of pressor amines may interfere profoundly with renal function. It seems pertinent that identical aortic medionecrosis and calcification can be elicited in the rabbit by renal injury with uranium nitrate and other nephrotoxic agents.[80, 81]

Raab,[78] on the other hand, believes in a direct "hypoxiating necrotizing action of catecholamines" on the arterial media. He found that advanced renal failure in human beings is accompanied regularly by a marked elevation of the blood catecholamine level,[82, 83] and he assumes that this factor may constitute a significant contributory element in the vascular lesions of patients

with kidney pathology. In support of his theory, Raab quotes Hueper's view[84] that the dependence of arterial necrosis upon severe renal excretory insufficiency is due to the toxic action of hypothetic "metabolites" accumulated in the blood. These metabolites may well be catecholamines but, under these conditions, one must first exclude the presence of excess parathyroid hormone.

However, it must be conceded that, while poisoning with endogenous or exogenous parathyroid hormone induces closely similar pathologic-anatomical alterations in animals other than the rat, including the canine and human species, the parathyroid gland may not necessarily respond as readily or as incisively to the stimulus of obstructive nephropathy or nephrectomy in other species.

Some of the cardiovascular lesions produced in the dog by various methods of renal injury or by bilateral nephrectomy bear a resemblance to the muscular injury induced in the rat by our procedures, although dogs show less involvement of the aorta and usually only minor degrees of calcification. Cardiovascular injury of this type was described by many groups of investigators, including those lead by Goldblatt,[85, 86] Winternitz,[87] Grollman and Muirhead,[88-91] Holman, [92, 93] and Corcoran and Page.[94, 95]

Goldblatt[86] points out that in the malignant phase of renal hypertension, even if it terminates fatally in as short a time as 48 to 72 hours, profound changes were observed in the blood vessels of the dog, monkey, rabbit, rat, sheep, and goat, and that the changes were similar to those seen in the terminal phase of human malignant hypertension. Generally speaking, the lesion consists of necrosis and fibrinoid degeneration of the media of small arteries and arterioles with or without perivascular inflammation characterized by the exudation of polymorphonuclear leukocytes and lymphoid cells. Most remarkable is Goldblatt's emphasis that "without accompanying significant disturbance of renal excretory function," the elevated intravascular pressure alone is not a sufficient condition for the production of the necrotizing arteriolar lesion, even if pronounced hypertension persists for many years.[86]

On the other hand, the parathyroid hormone is not necessarily implicated in every type of arterial necrosis following renal injury or emerging after bilateral nephrectomy. In fact, there is much evidence to the contrary, concerning particularly the periarteritis nodosa type of injury, localized usually in small arteries and arterioles, and believed by some to be a hypersensitivity reaction or a consequence of sodium accumulation in the tissues, produced by combining unilateral nephrectomy with saline administration and heavy dosages of DCA.[96, 97] In fact, it has been reported that thyroparathyroidectomy[57] enhances this vascular lesion.

A similar microscopic necrotizing vascular disease in small arteries of rats was seen by Koletsky[93] within a few days following infarction of the kidneys. Development of the lesions did not depend on hypertension or renal excretory failure, but was inhibited by total adrenalectomy. Koletsky considered injury to the kidney as the primary pathogenic factor, and he did not rule out the possibility that a "necrotizing substance" derived from the infarcted kidney was responsible for the vascular necrosis.

Like and Orbison,[99] who used a modification of this technique (infarction of one kidney and figure-eight ligature around the other kidney), obtained the same vascular injury in the parathyroidectomized rat and concluded that the pathogenesis of this microscopic lesion of small arteries is different from the medionecrosis of the arterial tree produced by our procedures.

Evidence against the existence of a specific musculotoxic factor in parathyroid hormone and supporting the importance of alterations in the calcium and phosphate metabolism is contained in the finding of equal or more intense "metastatic calcification" produced by A.T. 10[40] or vitamin D[100] in the absence of the parathyroid glands. Schelling[100] reported that parathyroidectomized rats are more susceptible to viosterol hypercalcification than normal rats, provided they are offered sufficient amounts of calcium and phosphorus in the diet. In the presence of a calcifying agent (in this case vitamin D) calcium phosphate is deposited in the tissues as the insoluble salt. Recent studies in our laboratory have confirmed that, in the rat, the removal of the parathyroids does not interfere with the development of severe calcification in the heart, aorta, stomach, and kidneys in vitamin D poisoning (unpublished data).

It has long been known that parathyroidectomized rats retain calcium and phosphorus,[101-103] and that they contain more calcium in their combined soft tissues than do intact control animals.[104] In the presence of optimal calcium levels, precipitation is favored by a high phosphorus intake. Whether degeneration and necrosis necessarily precede this form of calcification remains a moot point.

Orbison and his associates,[105, 106] working with nephrectomized dogs, found more pronounced vascular disease (microscopic necrosis in the media of small visceral arteries) when an "electrolyte" solution containing phosphate rather than 0.9 per cent saline was injected intraperitoneally. Since our findings in the rat suggested that the aggravation caused by phosphate administration might have been the result of stimulation of the parathyroid glands, Orbison and his associates[107] recently combined bilateral nephrectomy in the dog with simultaneous thyroparathyroidectomy and substitution of calcium by intraperitoneal injection with their "electrolyte fluid." Since, under these conditions, the absence of the parathyroid glands did not prevent the development of vascular injury, these investigators concluded that the pathogenesis of the arterial damage was different from that which we produced in the rat. This view might well be correct, yet one should not overlook the fact that, as a result of repeated injection of electrolyte fluid without subsequent removal, these reniprival dogs were accumulating both calcium and phosphate in their tissues and were in addition exposed to increasingly grave derangement of their mineral metabolism and fluid balance.

Recently Selye and his associates, in a series of papers that has been summarized in book form,[107a] reported that severe injury to the myocardium can be induced, among other means, by the daily combined administration, over a period of 12 days, of excessive dosages of inorganic phosphates such as NaH_2PO_4 and powerful synthetic corticoids, whereas neither phosphates nor corticoids alone, even in fatal dosage, were able to evoke any substantial cardiac lesions.

The term "electrolyte steroid cardiac necrosis" (ESCN) was proposed for this myocardial damage.

According to Selye[107a] (page 89), the parathyroid hormone "is not an obligatory prerequisite" for the development of ESCN, but it has a "conditioning influence." Selye's view of the aggravating influence of parathyroid hormone is based on unpublished data, quoted in his book[107a] (page 89), which purported to show that "after parathyroidectomy, the individual cardiac lesions tend to be smaller and more disseminated than under ordinary conditions and that large necrotic patches of the ESCN are rare."

This "conditioning influence" of parathyroid hormone would fit well into the concept that we have presented, but it could not be substantiated in our laboratory.[107b] Using Selye's technique of phosphate-corticoid poisoning, we found that parathyroidectomized rats were unable to cope with the enormous sodium phosphate load, since they succumbed within maximally 4 days of phosphate-corticoid or phosphate administration alone, whereas they tolerated the synthetic corticoid alone for the full experimental period of 12 days.

However, despite their short life span, parathyroidectomized rats under phosphate load, with and without the addition of the corticoid, almost invariably manifested the severest degrees of cardiac lesions (ESCN), even in instances where survival was less than 2 days. Contrary to the findings of Selye, therefore, the parathyroid hormone, under the conditions of his experiment, appeared to exert a protective influence, rather than an aggravating one. Moreover, similar severe cardiac lesions were induced by the administration of the corticoid alone. These findings, therefore, do not support the concept of mutual conditioning by exogenous electrolytes and steroids as a basic necessity for the development of this particular myocardial injury.

Lesions produced by either phosphate or corticoid administrations were indistinguishable, under the naked eye or histologically, from those designated as ESCN or "infarctoid necrosis" by Selye. However, even lesions emerging as early as 1½ days after the start of phosphate loading represented, primarily, areas of intense focal interstitial myocarditis consisting of infiltration with mononuclear cells that appeared to invade and destroy both muscle fibers and ground substance. Somewhat later, histiocytes and polymorphonuclear leukocytes entered the picture. A total of 70 rat hearts, presenting numerous macroscopically visible grayish-white patches (pinhead to rice-kernel size) on the pericardial and endocardial surface, were studied under the microscope. In no instance and in no stage of development (from 1½ to 12 days) did these grayish-white patches consist of larger areas of muscle necrosis surrounded by inflammatory cells that one might expect from the connotation "infarctoid necrosis." The picture was rather that of a diffuse necrotizing myocarditis.

The aggravation of this myocarditis by parathyroidectomy is at variance with our earlier observation that the removal of the parathyroids provides protection against similar myocarditis as well as against cardiovascular necrosis resulting from impairment of the renal excretory function.

In view of similarity of the gross and histologic appearance of the myocarditis and the apparent importance of phosphate accumulation and of min-

eralocorticoids under these divergent experimental conditions, the contrasting influence of parathyroidectomy is particularly interesting, although it does not necessarily indicate alternate mechanisms for the production of necrotizing myocarditis. In fact, the difference in experimental conditions can serve as an adequate explanation for the contrasting influence of parathyroid hormone. In instances of severe renal excretory impairment, as in our studies, an excess of parathyroid hormone will increase both calcium and phosphate levels in soft tissue by the continuation of internal mobilization from bones without the possibility of adequate elimination, and is thus deleterious. Under Selye's experimental conditions of external phosphate loading to the point of poisoning in rats with intact kidneys, parathyroid hormone protects by its phosphaturic action and is, in fact, essential for survival.

This interpretation is supported by the behavior of calcium and inorganic phosphate levels in the serum of intact and parathyroidectomized rats during phosphate and phosphate-corticoid administration.[107c] Under phosphate load, intact rats manifested no substantial elevations of serum phosphate levels, and high-normal or elevated calcium levels in the course of the first 4 days. Only after at least 1 week of phosphate-corticoid administration did we observe moderate and transient elevation of the serum phosphate concentrations, which were accompanied by dips from usually markedly hypercalcemic levels to normal or below-normal values. No substantial elevation of serum phosphate was obtained with phosphate administration alone, although hypercalcemia was observed.

Parathyroidectomized rats, on the other hand, starting with hyperphosphatemia and hypocalcemia, showed steep elevations of the phosphate level (15 to 20 mg. per cent) within the first 24 hours of phosphate or phosphate-corticoid dosage, and further cumulation with each successive 24-hour period. Terminal values on the third and fourth days reached 60 to 90 mg. per cent, with calcium values dipping simultaneously to 5 mg. per cent and lower.

Under phosphate load, the incidence and severity of myocarditis seemed to be related to the degree of hyperphosphatemia produced. Without substantial elevation of the phosphate level, severe myocarditis did not occur, and the earlier and the greater the rise in serum phosphate, the more severe the myocarditis.

Claims of specific antagonism for compounds or ions that inhibit the emergence of phosphate-corticoid-induced myocarditis, therefore, may be premature without determination of their influence on tissue phosphate levels in particular and on electrolyte metabolism in general. Protective action may be based on phosphaturic, generally diuretic, or even laxative action of the "antagonist," resulting in the diminution of "deleterious" electrolytes in the tissues and thus would represent a reduction of the eliciting stimulus rather than true antagonism.

By the same token, the aggravation of cardiac injury by certain compounds may be the result of electrolyte cumulation due to interference with excretory channels (for example, severe renal injury by chlorates), rather than a true synergism ("sensitization") at the tissue level.

Our observation that severe medionecrosis of the arterial tree could be produced within days and even in less than 24 hours in the absence of systolic hypertension goes beyond the recent data of Schaffenburg and Goldblatt.[108] These workers failed to confirm the finding of Byrom and Dodson[109] of necrotizing arterial and arteriolar lesions in the kidney as a result of repeated and sudden increases of systemic intra-arterial tension. Our findings demonstrate that an elevated blood pressure is not required for the production of arterial necrosis. However, this does not exclude the important contributory role of intravascular tension as suggested by the low incidence of lesions in the smaller circuit and also by the amazing protection of the aorta peripheral to mechanical compression by the vena azygos (FIGURE 28).

The presently widely accepted concept of the mechanism of experimental renal hypertension, as summarized by Floyer,[25] prompted us to consider the possible participation of the parathyroid hormone in the development of this hypertension. According to this concept, an extrarenal pressure mechanism operates in the chronic stage of hypertension, whereas a direct renal pressure substance plays little or no part in maintaining the blood pressure at this stage. This extrarenal pressure mechanism is closely linked with the adrenals and with salt metabolism and is inhibited by the intact kidney with normal circulation. It continues to operate after total nephrectomy, but is inhibited when normal circulation is restored to a structurally intact kidney. The same mechanism comes into operation and raises the blood pressure after total nephrectomy in previously normal rats. Although the parathyroid hormone was not previously implicated in this mechanism by others, the abolition of both cardiovascular necrosis and early hypertension in reniprival rats by prior parathyroidectomy suggests that the parathyroid hormone may contribute also to the development of renal hypertension.

The contrasting influence of DCA and cortisone upon survival time and cardiovascular and smooth muscle necrosis following standard renal injury and the complete reversal of the effects of these corticoids upon survival time in reniprival rats deserve further investigation. The inability of cortisone to support the development of renogenic muscular necrosis, as does DCA, is possibly linked to the phosphaturic action of the glucocorticoid,[110] which would tend to decrease the stimulus to parathyroid hyperfunction. This interpretation is in line with the results in the nephrectomy studies, where large dosages of cortisone equaled DCA in enabling the emergence of muscular necrosis, possibly because phosphate could not be removed under these conditions.

As to survival time, it is conceivable that the divergent effect of the glucocorticoid and mineralocorticoid upon the life span of reniprival rats is related to the protein catabolic effect of the former, which would be expected to accelerate potassium accumulation and, in general, to aggravate the uremic state. Studies on the behavior of the nitrogen, sodium, and potassium metabolism under our experimental conditions may provide an answer to this problem. The "antiuremic" and life-prolonging effect of DCA in nephrectomized animals was noted before us by other workers.[111-114] The poisonous effect of cortisone in acute renal excretory failure may be of considerable practical importance.

FIGURE 28. Thoracic aorta of rat *in situ* 10 days after standard injury. Note the sharp demarcation between severe necrosis, calcification, and aneurysmic dilation of the aortic arch and the thin, transparent, and normal-appearing thoracic aorta at the crossover point of the vena azygos. In all such instances the dilated descending part of the aortic arch was found overriding the tautly stretched vein, so that mechanical compression was exerted upon the aorta at this particular location. The black cardboard square was inserted for better illustration of the injured arch and the transparency of the normal thoracic aorta.[9] Reproduced by permission of *The Proceedings of the Society for Experimental Biology and Medicine.*

It is important to keep in mind the fact that the dosage of parathyroid extract necessary to produce this picture of severe cardiovascular necrosis in the rat is relatively small for this species, since the daily dose used most often in our studies (100 units) is the amount required to maintain the calcium level of the parathyroidectomized rat within the normal range for 24 hours. Our findings suggest that under appropriate stimulation the animal's own parathyroid glands can release quantities of hormone with many times this potency within the same interval of time.

Whatever the ultimate local events responsible for the death of muscle cells, a problem that still awaits elucidation, the fact that extensive disseminated muscular necrosis can be produced by partial or complete renal excretory failure within days in the rat and other species appears to be of considerable practical significance.

Obviously, there are numerous clinical conditions in which such a mechanism may be called into play. One may think here primarily of the vast group of renal diseases that entail acute or chronic excretory insufficiency and of numerous other disease entities in which the kidney is dominantly involved, such as malignant hypertension and toxemia of pregnancy.

The fact that cardiovascular necrosis is not encountered more frequently can be explained by therapeutic measures employed that tend to correct the severe metabolic derangements caused by the failure of renal function. This applies, for example, to peritoneal dialysis or "artificial kidneys" in experimental and clinical excretory renal failure which, by alleviating or abolishing the mineral and other metabolic disturbances, may remove the immediate cause of muscular necrosis and the stimulus for further excess production of parathyroid hormone. In those instances where correction is not attempted or difficult to achieve, necrosis may indeed be prominent.

Scholz,[115] for instance, reviewing the literature on myocardial calcification in human beings, concludes that the condition is not uncommon and that "deposition of calcium salts within the heart takes place only in dead or markedly deteriorated [tissue], never in healthy tissue." He suggests a possible "close interrelationship" with pathological conditions of the kidney. Similarly, Gore and Arons,[116] on the basis of a careful pathological study of 13 human cases of myocardial calcification at the Army Institute of Pathology, Washington, D. C., report that in all these instances "calcium deposits had been laid down on necrotic muscle fibers . . . . In 1 case there was a history of excessive intake of vitamin D. Azotemia occurred in all 12 of the remaining cases, being of renal origin in 11 and of prerenal origin in 1." The authors emphasize that necrosis seemed to be the *sine qua non* of myocardial calcification, and they speculate in their discussion on the causative role of renal injury.

Recently Wexler and Miller[116a] reported the production of "fulminating arteriosclerosis" of the Mönckeberg type and other "disease syndromes" in the rat by the chronic injection of nearly "therapeutic" amounts of adrenocorticotrophin (ACTH, 0.333 units per 100 gm. of body weight). Since adrenal corticoids, even in exorbitant dosages, fail to induce this picture even in the absence of advanced renal injury, the arteriosclerosis-producing effect

of ACTH could be explained only by postulating an "extra-adrenal" toxic action of this hormone.

In view of the significance of such a mechanism, the experiments of Wexler and Miller were repeated in our laboratory in 2 series, using ACTH dosages 3 times and 20 times higher than that indicated above, and for a period of 7 weeks.[116b] Rats of both sexes in several age groups were employed, including, in particular, "old discarded breeders," as was done by these authors.

In a total of 50 animals, all of which were carefully autopsied, utilizing a binocular loop and a dissecting microscope for close inspection of the heart and aorta, we were unable to find any cardiovascular or other conspicuous organic lesions. At the lower dose level the moist organ weights were not significantly different from littermate controls. With the higher dose, marked adrenal enlargement and thymic involution was a consistent finding.

Our inability to reproduce the "disease syndromes" described by Wexler and Miller, despite a threefold and a twentyfold increase in ACTH dosage, would seem to indicate that the "fulminating arteriosclerosis" seen by these authors had no relationship to the ACTH administration but was the result of incidental severe renal impairment in "discarded breeders," which is mentioned in their report as an aggravating factor.

The proposed existence of an adrenal cortex-parathyroid axis could provide an alternate explanation for the observations of Houssay[117] and others[118, 119] that extirpation of the pituitary is associated with cellular atrophy of the parathyroid glands and that administration of anterior pituitary extracts causes proliferation of parathyroid structures and a rise in serum calcium. These findings were considered evidence for the existence of parathyrotrophic hormone.[120] In fact, Anselmino and his associates[121] believe that they have demonstrated its presence in anterior pituitary extract. Today the concept of a direct anterior pituitary-parathyroid axis has been generally abandoned.[122]

In addition, excess release of parathyroid hormone under the influence of adrenal corticoids in the presence of renal excretory insufficiency could provide an answer to the advanced demineralization of the skeleton seen not infrequently in spontaneous and induced hypercorticism (Cushing's syndrome). This severe osteoporosis does not appear adequately accounted for by the protein catabolic effect of glucocorticoids.

Finally, the concept of an adrenal cortex-parathyroid axis may shed new light on the unquestionable amelioration sometimes achieved in severe hypertensive cardiovascular disease by bilateral adrenalectomy, even without decline of the blood pressure.[123, 124] Thus far, the possible presence of parathyroid hyperfunction in malignant hypertension has not been explored. If evidence for its existence is obtained, subtotal parathyroidectomy or chemical blocking of the parathyroid glands should prove therapeutically effective. It certainly would constitute a decidedly less dangerous and less mutilating procedure than total ablation of the adrenal glands.

The problem of the possible role of the parathyroid gland in severe hypertensive cardiovascular disease of higher animals and man is at present under study in this laboratory.

Summary

Using a method of standard renal injury (obstructive nephropathy), transient hypertension and severe disseminated necrosis and calcification were produced in the myocardium, the media of the arterial tree, and the muscularis of the gastrointestinal tract of the albino rat within 4 to 6 days. The gross and microscopic appearance of these lesions was illustrated.

Removal of the parathyroid glands provided complete protection against the development of this renogenic cardiovascular and smooth muscle necrosis. Since the protection of parathyroprival rats could be readily obviated by the administration of parathyroid extract, it was concluded that in intact rats excess parathyroid hormone released by the animal's own glands was essential for the production of the muscular lesions.

Indirect evidence was presented that excess production of parathyroid hormone as a consequence of renal impairment may be mediated through the adrenal cortex, and that mineralocorticoids are important for direct or indirect activation of renogenic hyperparathyroidism. In the absence of the parathyroid glands excessive dosages of desoxycorticosterone acetate (DCA) failed to produce cardiovascular and smooth muscle necrosis, whereas exogenous parathyroid hormone assured the emergence of typical lesions in the complete absence of mineralocorticoids even without the aid of renal injury.

Since the parathyroid glands appeared to be stimulated to release of excess hormone by phosphate retention and possibly other mineral metabolic derangements resulting from severe impairment of renal excretory function, it was reasoned that outright removal of both kidneys, if the animal survived for a sufficient period, should induce the same basic injury, and that reniprival muscular necrosis should be amenable to the same aggravating and attenuating influences.

It was found that albino rats survived bilateral nephrectomy without therapy usually for 3 to 4 days, and that the majority of such animals manifested the typical picture of cardiovascular and smooth muscle necrosis previously obtained with standard renal injury. Administration of parathyroid extract on the first and second days after nephrectomy resulted in dramatic enhancement of the incidence and, especially, in the severity of these lesions. A similar aggravating effect was achieved in nephrectomized as well as nephrectomized-adrenalectomized rats by injection of DCA and/or cortisone. On the other hand, rats deprived of their parathyroid glands prior to nephrectomy failed to develop any muscular necrosis even in the presence of excessive amounts of glucocorticoids and mineralocorticoids.

Since disseminated muscular necrosis could be produced by removal of the kidneys as readily as by standard renal injury, the existence of a renal "poison" appeared to be eliminated conclusively. The presence of a renal protective mechanism could also be excluded on the basis of the fact that parathyroidectomy provided complete protection against reniprival muscular necrosis. It also abolished early reniprival hypertension. However, hypertension was not a necessary accompaniment of renogenic or reniprival muscular necrosis.

In exploring further the adrenal cortex-parathyroid "synergism," it was

found in parathyroprival rats that, after simultaneous adrenalectomy-nephrectomy, the administration of exogenous parathyroid hormone still produced typical muscular necrosis. However, under these conditions adrenal corticoids did not enhance the muscular injury. These facts militate against a peripheral synergistic action of parathyroid and adrenal cortical hormones.

Experiments in rats adrenalectomized 10 days prior to nephrectomy and maintained on large daily dosages of cortisone and/or DCA indicated that in the presence of intact parathyroid glands either of these hormones, as well as their combination, permitted the emergence of typical muscular necrosis.

The relationship of adrenal cortical hormones to the parathyroid gland, as apparent from the findings in both renogenic and reniprival cardiovascular and smooth muscle injury, is believed to be consistent with the concept of an adrenal cortex-parathyroid "axis."

The pertinent literature is reviewed primarily in an effort to explore the ultimate mechanism of muscular necrosis emerging in the presence of excess parathyroid hormone.

The possible implications of our animal experimental observations upon the pathogenesis and therapy of human disease entities involving renal excretory insufficiency, hypertension, and cardiovascular injury are briefly considered.

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EFFECTS OF SEX STEROIDS ON LIPIDS*

Norio Higano, William D. Cohen, Roger W. Robinson

The Research Laboratory and the Medical Division, The Memoral Hospital, Worcester, Mass.

It has been established that the administration of male and female sex hormones in adequate dosage can influence profoundly the levels of serum lipids and lipoproteins.[1-9] Clinicians have observed that women are relatively immune to the development of coronary heart disease prior to the menopause[10,11] and lose this protection postmenopausally.[12,13] During the period of normal cyclic estrogen production in the young female, serum lipid levels are low. In contrast, abnormal serum lipid patterns and a greater incidence of coronary heart disease have been found in men[14-16] and in females with hypoestrogenic states, as in postmenopausal women[14, 15, 17] and in prematurely castrated women.[18] Such observations have indicated a possible relationship between estrogen metabolism and serum lipid levels.

Improvement of the serum lipids by the administration of estrogens has been demonstrated in both men and women. It is effective and well-tolerated in women, but estrogenic side effects in men have prevented the widespread clinical acceptance of this form of therapy for coronary atherosclerosis. The final results of well-controlled long-term studies now in progress[19,20] must establish statistically valid benefits in terms of decreased morbidity and mortality that are sufficient to outweigh the unpleasant side effects before potent estrogens can be accepted as a form of therapy for myocardial infarction in men. In an attempt to find an effective agent with minimal side effects in men, analogues of natural estrogens were studied. Although these substances had only slight estrogenic activity with potent lipid-shifting and antiatherogenic properties in experimental animals, the clinical results in men were disappointing because of significant estrogenic side effects.[21] Adequate androgen administration has been reported by others to have deleterious effects on the serum lipids.[7, 8] In the search for lipid-shifting agents that would be acceptable to men it was of interest to explore the lipid response to a minimal dose of a standard androgen, methyltestosterone. The availability of 17α-ethyl-17-hydroxy-19-nor-4-androsten-3-one (Nilevar†), a compound showing a wide separation of androgenic and anabolic activities, with only 6 per cent of the androgenic activity of testosterone propionate in rats,[22] prompted a study to determine whether its lipid-shifting properties were retained with its anabolic activity or diminished with its decreased androgenicity. SC-6582† (17α-methyl-17-hydroxy-5(10)-estren-3-one) a substance having estrogenic, androgenic, and progestational properties in experimental animals,[23] was also investigated. Finally, the effects of castration on the serum lipids of women who had undergone bilateral

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† Trademark of G. D. Searle & Co.

oophorectomy for surgical indications were observed. The results of some of these experiences with the serum lipid effects of estrogens in women and of androgens in men form the basis of this report.

Material and Methods

Clinical material. One hundred eighty-eight normal female employees from 21 to 70 years of age were selected at random from various departments of The Memorial Hospital for the base-line serum lipid studies after clinical evaluation to rule out cardiovascular disease. A large group of women bilaterally oophorectomized prior to age 45 have been studied, and the serum lipid data from 107 of these subjects are available. Seventy women were started either on 0.625 or 1.25 mg. of oral mixed, conjugated equine estrogens (Premarin*), and the dosage was increased at 3-month intervals. In order to avoid uterine bleeding only those subjects previously hysterectomized were increased above 1.25 mg. The purpose of this stepwise increase of dosage was to determine the quantitative relationship between estrogen dosage and serum lipid changes. Monthly clinical evaluations and serum lipid studies were obtained in all subjects. Papanicolaou vaginal smears were examined both before and during therapy to rule out the possibility of uterine cancer and to determine the estrogenic response of the vaginal mucosa.

Thirty-six men with coronary heart disease were divided into 3 groups of 12 each and, after at least 2 control serum lipid studies, the first group received 10 mg. daily of buccal methyltestosterone; the second, 10 mg. of oral Nilevar; and the third, 15 mg. of oral SC-6582. Administration of these compounds was continued for a trial period of 3 months, serum lipid studies and clinical evaluations being made monthly with special reference to the anabolic and androgenic effects of these compounds, as well as to any possible deleterious cardiovascular effects.

Methods. Blood samples were obtained from all subjects in the postabsorptive state and serum lipid determinations were performed as previously described.[5, 17] These determinations included total cholesterol, lipid phosphorus, and the cholesterol content of the α- and β-lipoproteins after ultracentrifugal separation. Total phospholipids, cholesterol/phospholipid (C/P) ratios and β-/α-lipoprotein cholesterol (β/α) ratios were calculated.

Results

The changes of total serum cholesterol in both aging normal women and oophorectomized subjects are shown in FIGURE 1. The base line for both groups is the average level of normal women in the third decade of life, 187 mg. per cent. Although the cholesterol levels increase with age in the normal women, the increases in the castrated women of corresponding ages were significantly greater. There was a larger difference in cholesterol levels (36 mg. per cent) between normal and oophorectomized women in the fourth decade than in the 51 to 70 age group (26 mg. per cent). The changes of the C/P

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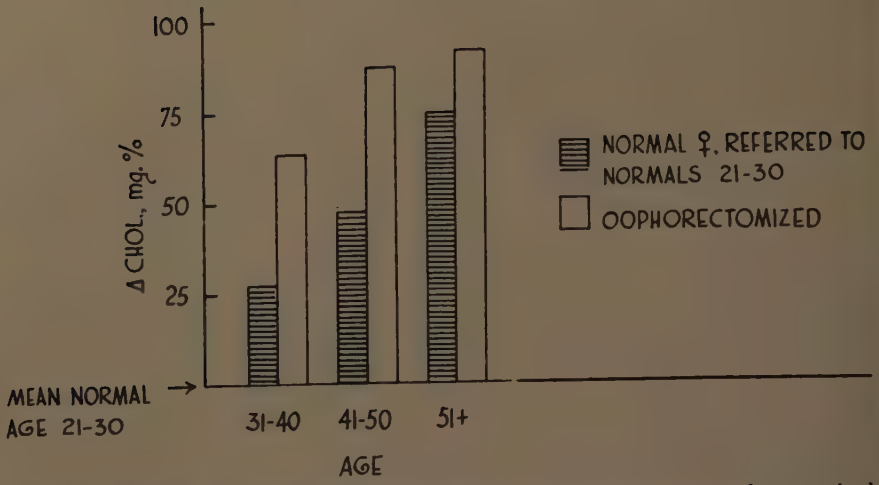

FIGURE 1. Changes in serum cholesterol in aging normal women and in oophorectomized subjects, age-matched to normals.

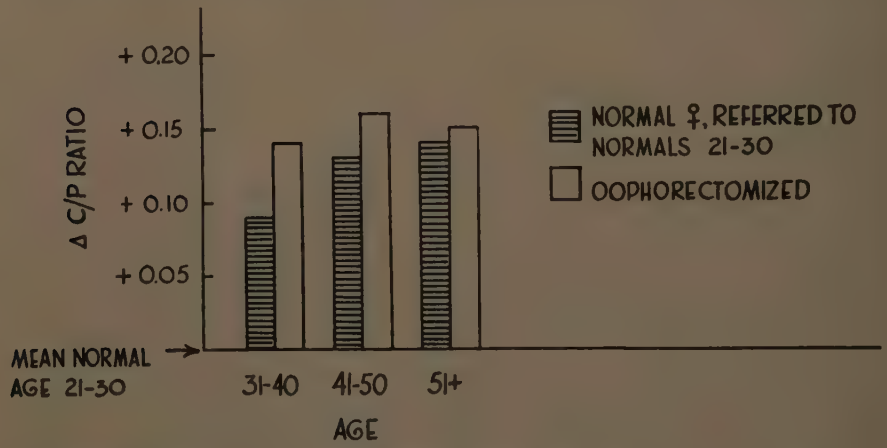

FIGURE 2. Changes in C/P ratios in aging normal women and in oophorectomized subjects, age-matched to normals.

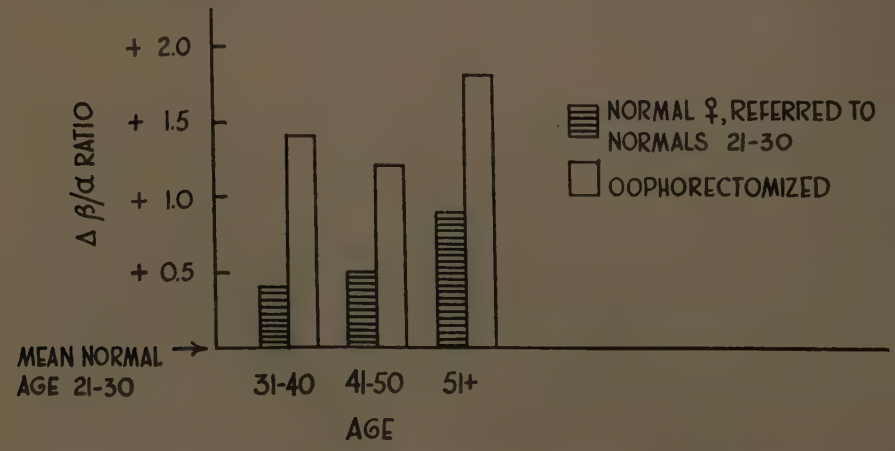

FIGURE 3. Changes in β/α ratios in aging normal women and in oophorectomized subjects, age-matched to normals.

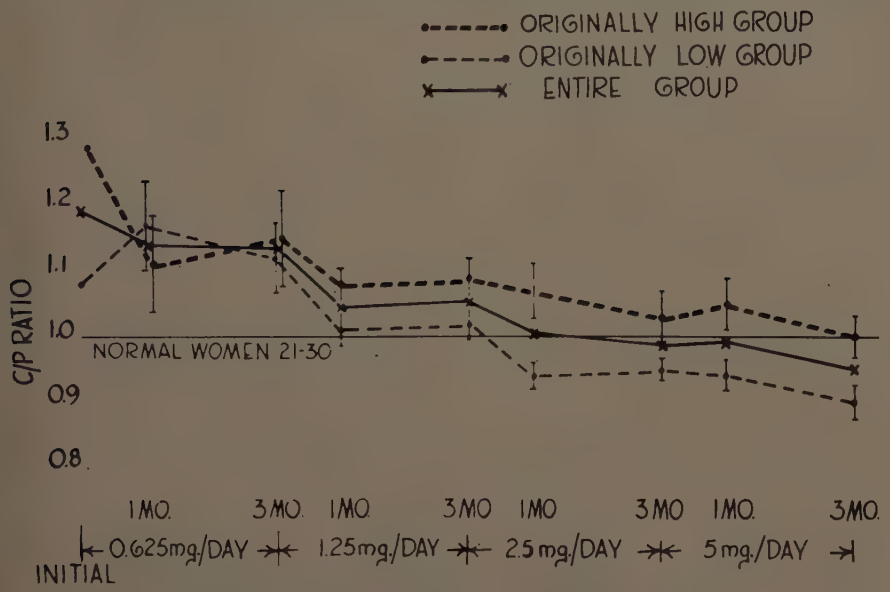

FIGURE 4. Response of C/P ratios to stepwise increasing Premarin dosage in women subdivided into initally higher and lower groups.

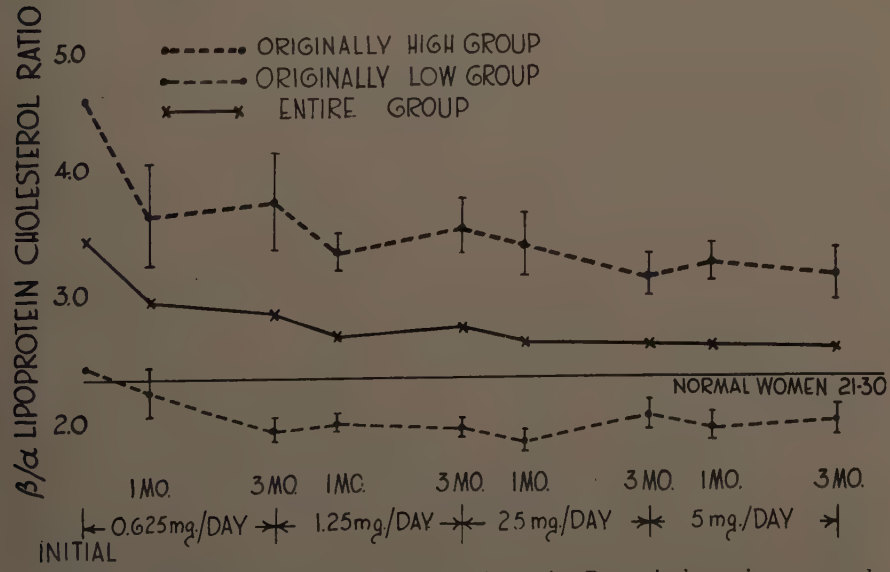

FIGURE 5. Response of β/α ratios to stepwise increasing Premarin dosage in women, subdivided into initially higher and lower groups.

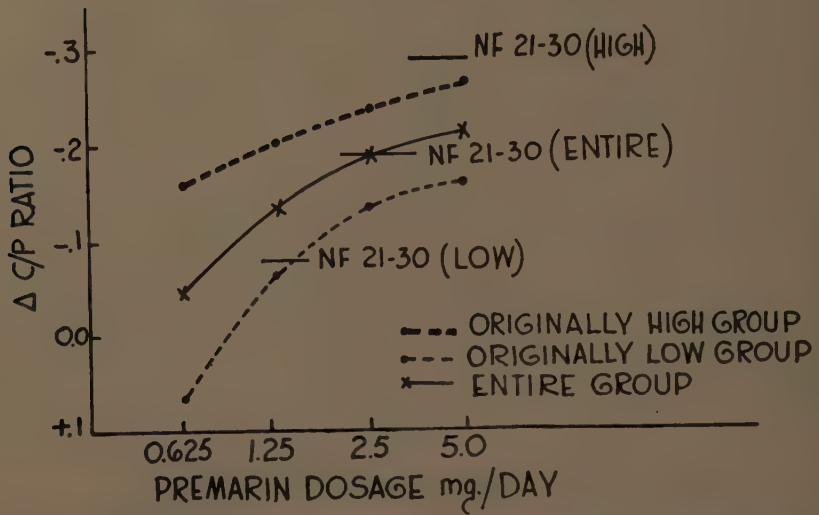

FIGURE 6. Changes of C/P ratio in response to increasing Premarin dosage (semilog plot of three-month data).

ratio with aging and castration compared to the 0.99 average found in normal young women are plotted in FIGURE 2. Despite a comparable rise with aging in both normal and oophorectomized women, the differentials were not as great as those shown in FIGURE 1, due to relatively greater increases of phospholipids in the castrated group. The profound differences in β/α ratios from the normal base-line level of 2.4 were similar in all age groups, as seen in FIGURE 3. There was a consistent rise of β/α ratios with increasing age in the normal subjects and a still greater increase in the castrated women. In the fourth decade this increase was more than 3 times that of the normal women.

The response of the C/P ratios to stepwise increases of Premarin is illustrated in FIGURE 4. On the basis of the mean result of the entire group it appeared that a 2.5-mg. dose was adequate to cause the C/P ratio to revert to that of normal young women, but a bimodal response was found. The subjects were segregated into those whose pretreatment C/P ratios were either above or below the group mean. It then became clear that in the initially more normal group the extent of response was less, but the desirable level (that of normal young women) was attained with the relatively small 1.25-mg. dose. Despite the greater response of the initially more abnormal group at all dosages, a 5.0-mg. daily dose was required to reach the level of normal young women. The β/α ratio data in these women also showed a bimodal response, as depicted in FIGURE 5. Both groups responded to 0.625 mg. of Premarin with decreases in the β/α ratio. Although the change was again greater in the initially higher group, even a 5.0-mg. dose did not lower the ratio to the ideal level.

A graphic illustration of the C/P ratio data as changes from the initial levels

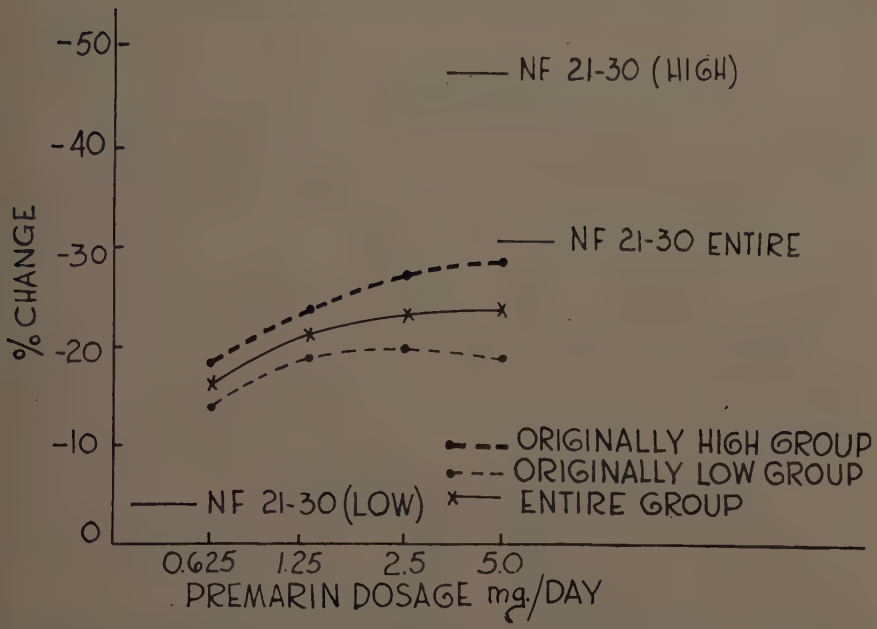

FIGURE 7. Changes of β/α ratio in response to increasing Premarin dosage (semilog plot of three-month data).

plotted against stepwise increments of Premarin dosage is shown in FIGURE 6. The greater response of the originally high group is apparent at all dosage levels and the response increases with each increment in dosage. A similar dose-response curve, using the changes of β/α ratio as a measure of lipid-shifting effect, is presented in FIGURE 7. Essentially maximal change in the lower group was attained with the 1.25-mg. dosage level but, in the higher group, 2.5 and 5.0 mg. daily produced further lowering of the β/α ratio.

The structural formulas of the 3 compounds administered to men in this study are depicted in FIGURE 8. The close chemical similarity of these substances is apparent. The lipid-shifting effects of these compounds in 3 months are shown in FIGURE 9. In the group on 10 mg. daily of buccal methyltestosterone there was only an insignificant increase in the serum cholesterol and no change in the phospholipids, resulting in a practically unchanged C/P ratio. Nevertheless, the moderate increase of the β/α ratio was highly significant. Clinically, these subjects suffered no progression of their cardiovascular disease, and weight gain and increase of sex functions were minimal, but 7 of the 12 men claimed an increased feeling of well-being. In the group receiving 10 mg. of Nilevar there was a tendency to an increase of the serum cholesterol and a decrease of the phospholipids, resulting in a statistically significant increase of the C/P ratio. The increase of the β/α ratio to nearly double the pretreatment level was highly significant. The average weight gain of these men during the 3-month period was 4 pounds, and one half of the group allegedly

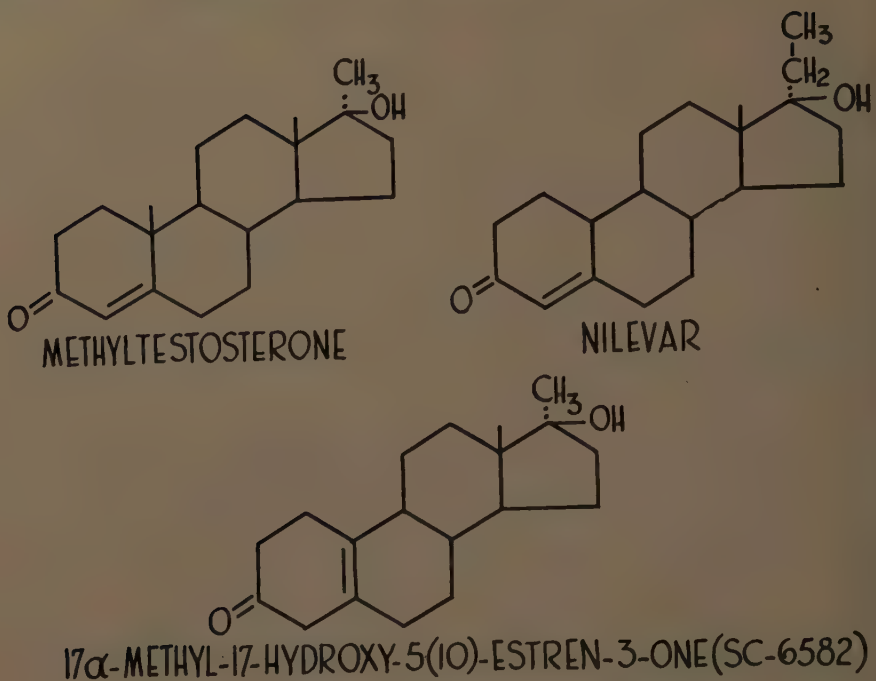

FIGURE 8. Structural formulas of compounds studied.

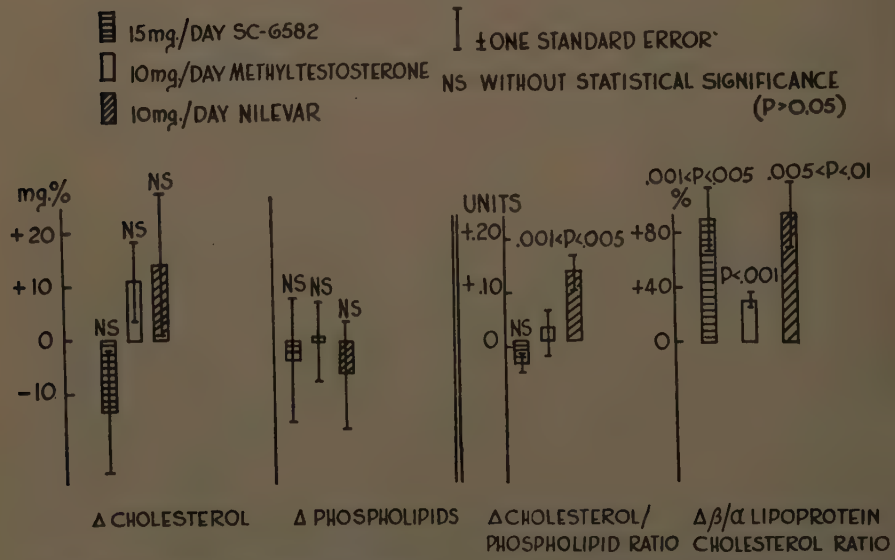

FIGURE 9. Serum lipid changes in men after three months of androgen administration.

enjoyed an increased sense of well-being, but there was only slight increase in sex functions. In the last group of men, who received 15 mg. of SC-6582, a highly significant increase of the β/α ratio was observed, and this was of the same magnitude as that of the Nilevar group. However, the change of total cholesterol, although not significant, was in the opposite direction, resulting in an insignificant decrease in the C/P ratio. There was an average weight gain of 2 pounds, with no effect on sex functions or any significant subjective changes.

Discussion

The data of the present study offer evidence for the importance of the postmenopausal decrease in ovarian function as at least a partial explanation for these lipid changes. The "worsening" of the total cholesterol, C/P ratio, and β/α ratio in the women oophorectomized early in life (under 40 years of age) was found to be similar in degree to that in the normal postmenopausal women of more advanced age in whom estrogen production is likewise decreased.[24] These results suggest the possible added risk of early castration in women, since preliminary evidence from this laboratory suggests an increased incidence of cardiovascular disease in these women. If bilateral oophorectomy must be performed, prophylactic estrogen therapy should be considered on a long-term basis unless otherwise contraindicated. The potential hazard of estrogen carcinogenesis in humans has not been borne out by the clinical findings of Henneman and Wallach[25] in women with postmenopausal osteoporosis treated for many years.

The dependence of the serum lipid response to estrogen therapy in women upon their pretreatment levels, described in an earlier publication,[17] has been recently confirmed by Marmorston *et al.*[26] Thus, patients with more nearly normal serum lipid levels require only small doses of estrogens to attain levels comparable to those of normal young women, a C/P ratio of 0.99 or less, and a β/α ratio of 2.4 or less. Since such low dosages produce minimal side effects, the prophylactic administration of estrogens to essentially all postmenopausal women may be considered as a practical measure. In the group of women with initially more abnormal serum lipids 9 months of estrogen therapy, with the last 3 months at 5.0 mg. of Premarin daily, was required to decrease the C/P ratio to the ideal level. Although this appeared to be related to the increasing dosage of estrogen, the importance of duration of therapy cannot be excluded. Studies of long-term administration at each dosage level are now in progress to evaluate this point. On the other hand, the decrease of the β/α ratio to 3.4 during this period was insufficient to attain the average level of normal young women. The reason for this is not apparent.

The gross changes in serum cholesterol, phospholipids, and hence in C/P ratio following administration of 50 mg. of methyltestosterone[7] did not occur at the 10-mg. dose level of this study, although there was a suggestion of an increase in cholesterol. The sensitivity of the β/α ratio to hormone administration was again borne out by a significant rise after 3 months of minimal androgen dosage. The serum lipid changes with Nilevar were of interest

because of their similarity to those produced by large doses of methyltestosterone despite the clinical dissociation of the androgenic and anabolic effects of Nilevar. The great increase of the β/α ratio was due mainly to an increase of β-lipoprotein cholesterol. A similar rise of β-lipoprotein was found by Sachs, of Weston's group.[27] The serum lipid changes produced by SC-6582 are noteworthy in that the fall in cholesterol, although highly variable, resembled that produced by conventional estrogens. The increase of the β/α ratio was consistent with an androgen effect, but this change was due chiefly to a decrease of α-lipoprotein cholesterol with only a slight rise of the β fraction, in contrast to the effects of Nilevar and of methyltestosterone, which were primarily increases of β-lipoprotein cholesterol. Thus, this compound had serum lipid effects of neither conventional androgens nor estrogens, despite some apparent anabolic activity, as reflected in weight gain. Our clinical experience with male patients given synthetic analogues of both estrogens and androgens and potent androgens at low dosage have revealed interesting effects on the serum lipid patterns, but the goal of desirable lipid changes without unfavorable side effects has not been achieved.

Summary

Increases of serum lipids, including total cholesterol, cholesterol/phospholipid ratios, and β-/α-lipoprotein cholesterol ratios were found in aging women, but such increases were more pronounced in castrated women of comparable age.

A bimodal serum lipid response to stepwise increasing estrogen dosage in women was observed, based upon the levels of their control serum lipid studies. Thus those with initially higher levels required a larger dose than did those with initially lower values for correction of their serum lipid abnormalities.

Short-term administration of three different androgens to men increased the serum β-/α-lipoprotein cholesterol ratios in each group. These changes in the groups treated with methyltestosterone and Nilevar were similar, in that they were chiefly due to increases of the β-lipoprotein cholesterol fraction. In contrast, the changes in the group treated with SC-6582 were due primarily to decreased α-lipoprotein cholesterol.

Acknowledgment

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THE SERUM LIPID PATTERN IN HYPERTHYROIDISM, HYPOTHYROIDISM, AND CORONARY ATHEROSCLEROSIS*

Richard J. Jones, Louis Cohen, Howard Corbus
Department of Medicine, The University of Chicago, Chicago, Ill.

The idea that atherosclerosis occurs prematurely and with greater intensity in patients with myxedema or cretinism is widely held. Scattered autopsy reports of isolated untreated cases that occurred more than 50 years ago are quoted to support this view, but these reports leave much to be desired. Microscopic findings in the lesions were poorly described, and these authors were much more intrigued by the more severe and dramatic changes observable in the thyroid gland, the skeleton, the skin, the muscles, and elsewhere. In examining each case reported to support this thesis we could find no convincing case of uncomplicated myxedema or cretinism where coronary or other intimal atherosclerosis was severe or premature. For example, Bourneville's (1903) oft-cited 36-year-old cretin was said to have only one isolated 2-cm. atheromatous plaque in the thoracic aorta: hardly an impressive lesion. Hun and Prudden (1888) reported autopsies on two 55-year-old myxedematous women: in one "the blood vessels throughout the body so far examined were the seat of endarteritis, with more or less atheromatous degeneration. The coronary arteries and their branches show a moderate degree of chronic endarteritis," and in the other no mention was made of arterial disease. Other cases, such as Heine's (1924), were hopelessly complicated by hypertension, nephritis, and other disorders that we accept today as intensifying atherosclerosis. In fact, Fishberg (1924) reported a case of myxedema in a young man of 21 who showed extensive atherosclerosis, plus hypertension and nephritis, to emphasize the kidney lesions so often found with athyreosis in the human (Scholz, 1906) and in experimental animals (Goldberg, 1927). The most significant fact is that in the definitive reviews of 106 collected cases of cretinism (Scholz, 1906) and over 50 cases of primary myxedema (Committee of the Clinical Society of London, 1888) made before treatment by thyroid extract was introduced, neither the frequency nor severity of atherosclerosis was considered remarkable.

More recently, Blumgart (1953) has reported his experience with 8 cases of hypothyroidism induced for the alleviation of congestive failure due to rheumatic heart disease, of whom 4 received no thyroid extract. Much was made of the fact that no coronary occlusions and only occasional arterial narrowing were found in these cases; the untreated patients aged 28, 44, 46, and 63, all had some atheromatous lesions, although these were said to be unremarkable. All we can conclude from the clinical pathological data is that, while coronary intimal atherosclerosis may occur with the usual frequency in the presence of myxedema, it is neither universally nor strikingly increased beyond the expected degree of severity; it is not complicated by coronary thrombosis or occlusion with undue frequency. There is thus reason to doubt that the

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atherogenic potency of the hyperlipemia associated with hypothyroidism is anything like that seen in essential hypercholesteremia with xanthoma tendinosum.

The situation regarding the lowered incidence of coronary disease in hyperthyroidism is hardly more definite. Just as myxedema occurs more often in the older age group where coronary atherosclerosis would be expected in high incidence, hyperthyroidism is a disease of younger people, among whom the incidence would normally be less. As in the case of myxedema, no significant number of hyperthyroid patients go untreated today for any length of time. Even more than myxedema, hyperthyroidism favors the female sex which, we have good reason to suspect, has other hormonal protection against at least the coronary type of atherosclerosis. In addition, patients with chronic hyperthyroidism also suffer a negative caloric balance, which we know discourages atheromatous development (Wilens, 1947) and is most difficult to exclude from consideration.

An autopsy study of 25 years ago concerned with the purported myocardial lesions associated with hyperthyroidism made an incidental report on the frequency of coronary atherosclerosis in a group of 35 patients with exophthalmic goiter (Weller *et al.*, 1932). An unstated number of hearts were excluded from the series because of evident gross coronary and other heart disease. Comparing the histopathology of the remaining 35 hearts with that seen in an equal number of carefully matched euthyroid control hearts, no outstanding difference was found, except that myocardial fibrosis, endocardial sclerosis, cellular infiltration, and myocardial hyperplasia were perhaps more frequently associated with hyperthyroidism. Microscopic atherosclerosis of the smaller ramifications of the coronary arteries was found with an equal prevalence (45 per cent) in both hyperthyroid and control groups.

The reduction of hypothyroidism from its position of prominence as a factor in atherogenesis is rather difficult for some to accept in view of the often substantial associated hypercholesteremia. It may well be that the hypotension and general reduction of tissue metabolism, and perhaps the intimal permeability, in myxedema serve to nullify what might otherwise be the atherogenic effect of its hypercholesteremia. On the other hand, could it be possible that the hypercholesteremia of myxedema is somehow different, and perhaps less atherogenic, than that seen in coronary disease or essential hypercholesteremia? We have presented some data that suggest that there is a qualitative difference between the hypercholesteremia of myxedema and that seen in coronary disease (Jones *et al.*, 1955).

Materials and Methods

Fasting serum was obtained on untreated patients in the University of Chicago Clinics who manifested hypothyroidism, hyperthyroidism, or coronary artery disease. Over a 3-year period samples were obtained on 25 untreated hyperthyroid patients, of whom 21 were female. Of these patients 3 had essential hypertension, 1 had the anginal syndrome, and 2 had malignant exophthalmus.

In the same period, sera were obtained from 17 patients with untreated

hypothyroidism, of whom 10 were female and 7 were male; 2 were in congestive heart failure, 1 was troubled with the anginal syndrome, 1 had hypertension, and 2 more had significant cardiomegaly. Thus, roughly one-third of these patients had suggestive evidence for heart disease, although none had an electrocardiographic pattern even suggestive of previous myocardial infarction.

From a large group of patients with coronary artery disease a few patients were selected to be matched with the hypothyroid group as to sex, age, and serum cholesterol level. In order to match the high serum cholesterol levels seen in certain hypothyroid patients it was necessary to include 1 man with a previous myocardial infarction and xanthoma tendinosum et tuberosum and 2 women who had as yet manifested no evidence of coronary disease, but who had xanthoma tendinosum in one case and xanthoma planum in the other. One myxedematous woman, 33 years old, could not be matched with any coronary disease patient, but was matched by a 25-year-old woman with comparable cholesterol level and a presumptive family history of coronary disease. The remainder of this matched group all had uncomplicated coronary artery disease, either proved myocardial infarction or a classic anginal syndrome. Except for 3 cases, the cholesterol levels matched within ± 30 mg. per cent. Except for 4 cases, the ages agreed within 6 years.

Duplicate samples of sera obtained from these patients before treatment were extracted in alcohol:ether as described by Bloor (1943) and analyzed in duplicate for total lipids, total cholesterol, and lipid phosphorus. A third 5-cc. sample was analyzed for lipoproteins by a modification of the heavy-density technique described by Lewis and Page (1953).

Results

In an effort to determine which lipid fraction was most influenced by thyroid activity we compared the two thyroid groups with regard to both chemical and physical fractions. In FIGURE 1 it can be seen that all of the chemical measurements, total lipid, total cholesterol, and phospholipid show about a twofold increase. Comparison of the ultracentrifugal data shows that this increase is due largely to an elevation of the low-density (beta) lipoprotein fraction and the associated faster rising components comparable to Gofman's S_f 12 to 20 and 35 to 100. The high-density ($alpha_1$) lipoprotein difference is the only one not significant statistically.

It is worthy of note that in the hyperthyroid group there were 10 patients with $-S_{1.21}$ 40 to 70 greater than 50 mg. per cent, and 7 had $-S_{1.21}$ 100 to 400 lipoproteins above 40 mg. per cent. Gofman believes that these fractions are atherogenic. These patients included the 2 with malignant exophthalmus, 2 with essential hypertension, and 1 with the anginal syndrome; thus, it would not appear that thyroid hyperactivity necessarily abolishes an elevation of these lipoproteins.

Examination of the serum lipid pattern in myxedema patients reveals no feature that distinguishes it by these techniques from that seen in coronary artery disease or hereditary hypercholesteremia. When sera, matched for cholesterol level, from patients of similar age and sex, are grouped and com-

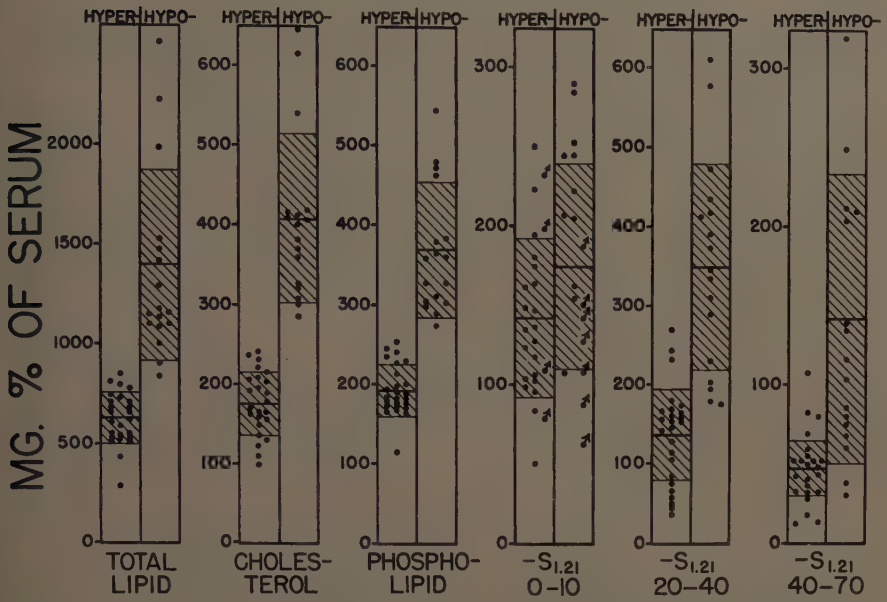

FIGURE 1. Comparison of the lipid values in 25 hyperthyroid patients and 17 hypothyroid patients. Scattergrams for the concentrations of total lipid, cholesterol, phospholipid, and various lipoprotein fractions. The sex of male patients is indicated by the small arrow attached to the points in the $-S_{1.21}$ 0 to 10 fraction, where sex hormones may exert some influence. The shaded portion of each bar represents one standard deviation on either side of the mean.

pared, the mean difference between the other chemical lipid fractions is not significant. However, when we examine the lipoprotein patterns (FIGURE 2), there is a statistically significant difference between the concentration of low-density (beta) lipoprotein, whereas lower-density lipoproteins with flotation rates of $-S_{1.21}$ 40 to 400 and the high-density ($alpha_1$) lipoproteins show no significant difference. The latter do vary, although not significantly, in a direction opposite to the low-density $-S_{1.21}$ 20 to 40 fractions, so that the difference between the ratios of $(-S_{1.21}$ 20 to 40$)/(-S_{1.21}$ 0 to 20) in the 2 groups is even more impressive.

It would seem appropriate in this monograph to speculate upon just what might produce a difference of this magnitude and suggest what course future research on this question might profitably take. This difference is a relative one and does not imply that atherosclerosis can be differentiated from myxedema by its ultracentrifugal pattern. These data do indicate, however, that the mean beta-lipoprotein concentration is lower in myxedema than in coronary artery disease for the same degree of hypercholesteremia. Since all the other factors are approximately the same, it suggests conversely that the beta-lipoprotein in myxedema is more heavily laden with cholesterol than that in coronary atherosclerosis. At the time we originally presented these data, no further speculation was attempted, but since then further chemical analysis of

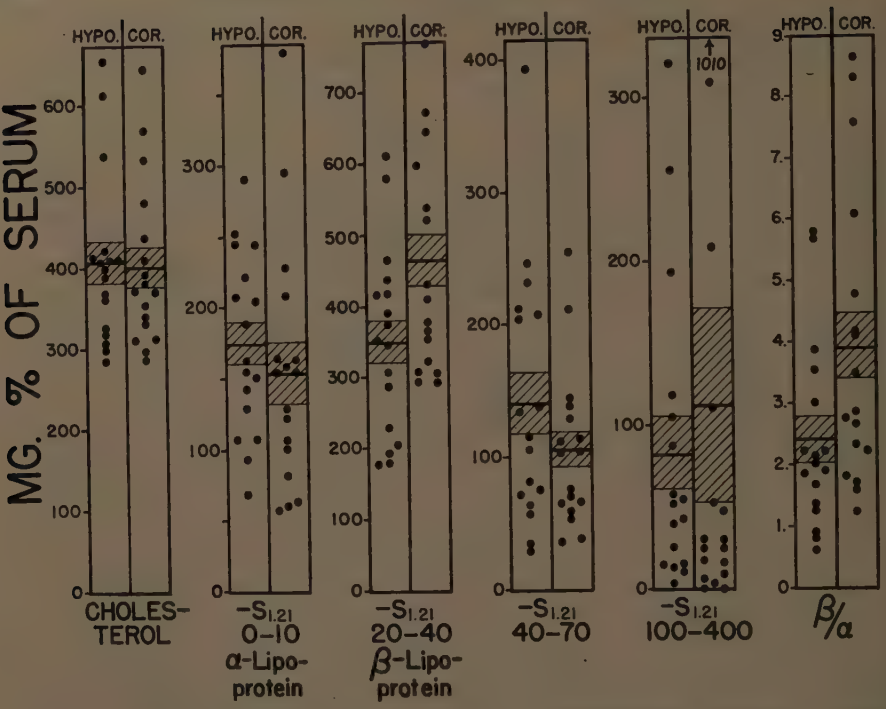

FIGURE 2. Comparison of the serum concentrations of cholesterol and lipoprotein species in a group of 17 hypothyroid patients, each matched as to age, sex, and serum cholesterol level with a coronary artery disease patient. The shaded portion of each bar represents one standard error on either side of the mean.

the various lipoprotein fractions done in other laboratories allows us to estimate roughly how great a disturbance in partition of the major peak of the low-density lipoprotein would occasion this difference.

From the recent work of Eder *et al.* (1955) it was noted that the lipid partition of the lower-density beta-lipoprotein as measured by the ultracentrifuge was not a consistent entity in all clinical states, particularly in patients with biliary cirrhosis. This suggests that greater concentrations of other lipids are perhaps present in this fraction under varied circumstances. More recently, Kunkel and Trautman (1956) have demonstrated that an alpha$_2$-lipoprotein is present in the region of our $-S_{1.21}$ 40 to 70. (This alpha$_2$-lipoprotein is different in centrifugal characteristics than that fraction so designated by Lewis and Page, 1953.) It is occasionally difficult to separate completely from the beta-lipoprotein ($-S_{1.21}$ 20 to 40) centrifugally, yet is distinct in its electrophoretic mobility and in the fact that chemically, while incompletely described, it is presumably higher in neutral fat and has a lower cholesterol lipid phosphorus ratio than the beta-lipoprotein, though not as low as the alpha$_1$-lipoprotein ($-S_{1.21}$ 0 to 10). Furthermore, Kunkel and Trautman reported that in a few patients with coronary disease this material was to be found in high concen-

tration, though the numbers of both control and patient sera were too few to allow any definite conclusions in this regard.

There is some doubt as to whether this alpha$_2$-lipoprotein can be equated with the "pre-beta-lipoprotein" observed on filter paper electrophoresis by Dangerfield and Smith (1955) and confirmed by Besterman (1957). This "pre-beta-lipoprotein" traveled ahead of, but overlapped, the main beta peak and was found in high concentration in patients with coronary disease by both of these authors; it was found to be absent in an unspecified number of sera from patients with myxedema by Dangerfield.

Let us suppose that in atherosclerosis there is an abnormal amount of a low-density lipoprotein that is somewhat dilute in cholesterol, compared to the "normal" beta-lipoprotein and that floats a bit ahead of the beta peak, often never really getting away from it in the centrifuge; perhaps this is the "pre-beta-lipoprotein," which has not yet been characterized in the ultracentrifuge. In myxedema, where the hyperlipemia seems to be explained simply by sluggish transport and excretion (Rosenman *et al.*, 1952), as well as decreased synthesis of cholesterol and, presumably, lipoproteins, we might hope to find an excessive accumulation of the "normal" beta-lipoprotein in the serum, which is relatively undiluted by this abnormal lipoprotein.

Let us further assume that all of the normal and abnormal beta-lipoproteins will be included in the combined total of lipoproteins moving in the range $-S_{1.21}$ 20 to 40 and $-S_{1.21}$ 40 to 70. The faster-rising lipoproteins are of less significance as bearers of cholesterol and, in any case, would cancel out in equating mean values in our two groups. What we have done by thus comparing the coronary disease group with the myxedema group of comparable cholesterol level may be expressed in the equation

$$(a) \times A + (b) \times B + (c) \times C = (a)A' + (b)B' \qquad (1)$$

where a, b, and c are the concentrations of cholesterol in the heavy-density (alpha$_1$), low-density (beta) and abnormal low density (pre-beta?) lipoproteins; A, B, and C are the mean concentrations in mg. per cent of heavy, low-density, and the excess of abnormal low-density lipoproteins in the coronary sera; and A' and B' stand for the same quantities in the myxedema sera. We can derive A, A', B', and $(B + C)$ from our data, and from the literature we can assume reasonable figures for a, b, and c. There is general agreement that the heavy-density lipoproteins carry about 20 per cent cholesterol, although this may apply only to normal serum. Havel *et al.* (1955) originally assumed that the low-density (beta) lipoprotein carried 30 per cent cholesterol, whereas other workers have found 40 per cent (Oncley, 1954) to 55 per cent (Besterman, 1957), using different methods of separation. Perhaps the best value to apply here is the 47 per cent cholesterol found by Bragdon *et al.* (1956), using ultracentrifugal separation. There is no definite figure for our hypothetical abnormal lipoprotein unless we use Besterman's estimate of 34 per cent for his pre-beta. This might be too high, in view of the value of 22 per cent found by Bragdon *et al.* (1956) for lipoproteins >1.019 in density, which were admittedly inhomogeneous. If we assume an intermediate value of 28 per cent, what

excess of this abnormal lipoprotein must occur in the material $-S_{1.21}$ 20 to 70 of the coronary sera to explain our data, assuming no excess was found in the myxedema group? Substituting these percentages in EQUATION 1

$$0.20A + 0.47(570 - C) + 0.28C = 0.20A^1 + 0.47B^1 \quad (2)$$

then if, from our mean values $A = 155$, $B + C = 570$, $A' = 175$, and $B' = 493$, we have:

$$31 + .28C - .47C + 260 = 35 + 227 \quad (3)$$

or

$$-.19C = -29, \text{ and } C = 153 \text{ mg. per cent} \quad (4)$$

Thus, of the total lipoprotein contained in $-S_{1.21}$ 20 to 70 of the coronary sera, 27 per cent would be this abnormal lipoprotein. It is interesting that Besterman (1957) found that his coronary patients had a pre-beta/beta ratio of 0.43, as opposed to a ratio here of 0.37. If we assume that our value B actually includes a small amount of pre-beta on both sides of EQUATION 1, the agreement becomes even better, so that our data would fit the hypothesis that an abnormal lipoprotein, perhaps related to the "pre-beta-lipoprotein" of Besterman, is present in coronary atherosclerosis to a greater extent than in myxedema and to a degree which is compatible with the assumption that the myxedema subjects have an excessive accumulation of normal low-density (beta) lipoprotein.

How do we know that the abnormality does not reside in the alpha$_1$-lipoprotein? This question is all the more disturbing because Eder (1957) has pointed out that the only abnormality recognized in several studies, employing Cohn fractionation and electrophoresis to compare coronary patients' sera and control sera, has been a decrease in concentration as well as absolute amount of cholesterol in the alpha-lipoprotein. Could the heavy-density alpha-lipoprotein of coronary patients' serum, being more dilute in cholesterol than in normal and myxedema sera, explain our findings? Employing the same sort of substitutions, and again assuming that myxedematous lipoproteins are normal in cholesterol concentration, the coronary group's alpha$_1$-lipoproteins would have to be so abnormal as to be carrying no cholesterol, a possibility which we find unacceptable. The possibility remains that there could be, in coronary patients' sera, abnormalities of both high- and low-density lipoproteins, but this need not be postulated: to explain our findings the presence of a substance in the region of $-S_{1.21}$ 20 to 70, having the approximate cholesterol concentration of Dangerfield's and Besterman's "pre-beta-lipoprotein," which occurs at greater concentration in coronary patients' sera than in myxedematous sera, is both plausible and sufficient.

This is not a direct proof that an excess of such a unique lipoprotein exists in coronary artery disease serum, as opposed to that of myxedema; we feel, rather, that it is suggestive. As implied elsewhere in these pages by Lindgren, the inhomogeneity of the low-density lipoproteins needs further study. We venture to predict that, when simpler means of examining the fractions of the

low-density lipoprotein are developed, the lipoprotein pattern of the post-coronary patient will be shown to differ substantially from that of the uncomplicated myxedema patient by its greater amount of a lipoprotein species, contaminating the low-density (beta) lipoprotein of ultracentrifugal definition, which is lower in cholesterol concentration than the predominant low-density lipoproteins.

Summary

Review of the historical evidence relating coronary atherosclerosis to myxedema suggests that there is no significant aggravation of intimal atherosclerosis by the latter. The incomplete disassociation between hyperthyroidism and coronary artery disease is as well explained by age and sex factors as by any differences in serum lipids.

Serum lipid chemistries and serum lipoproteins determined by the heavy-density technique of ultracentrifugation were compared in 25 hyperthyroid and 17 hypothyroid patients. Certain low-density lipoproteins in the range of $-S_{1.21}$ 40 to 400 were found to be somewhat increased in several of the hyperthyroid patients, but there was the anticipated difference between these groups in all the lipid moieties, except for the heavy-density lipoproteins.

In comparing the lipid patterns of the myxedema group with those of a coronary artery disease group, matched with regard to sex, age, and serum cholesterol level, no significant differences occurred in the mean values of the various fractions except in the low-density fraction ($-S_{1.21}$ 20 to 40), which was greater in the coronary group. Making certain assumptions and employing published data on the composition of alpha-, beta- and Dangerfield's "pre-beta-" lipoproteins, differences among these groups could be explained by the presence of an excess of a lipoprotein in coronary disease serum traveling with the low-density lipoprotein but having approximately the same cholesterol concentration and occurring in the same proportion as reported for the "pre-beta-" lipoprotein.

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THE PANCREAS AND CARDIOVASCULAR DISEASE

Charles W. Mushett
Merck Sharp & Dohme Research Laboratories, Rahway, N. J.

In this paper I propose to review some findings that have contributed to our present state of knowledge on the pancreas as it relates to the development of cardiovascular disease and to present a few original observations of related interest from this laboratory.

The well-substantiated observation that degenerative vascular disease occurs more frequently in persons with the pancreatic disease, diabetes mellitus, than in the general population has prompted inquiry into the nature of this association. Cardiorenal-vascular complications may account for about 75 per cent of total deaths in diabetics.[1] This high mortality figure assumes added significance with the consideration that there are more than two million diabetics in the United States and an estimated twenty million in the world.[1] In this disease virtually all components of the vascular system are involved, including the arteries, arterioles, capillaries, and venules. There appears to be general agreement that diabetes or a related factor not only accelerates the onset but also increases the incidence and severity of arteriosclerosis and arteriolosclerosis.[2] From the clinical standpoint the most important vessels thus involved are the medium-sized arteries of the heart, brain, and lower limbs, and the arterioles of the kidney. The diabetic, furthermore, develops certain capillary lesions of the renal glomeruli and retina that are virtually characteristic of this disease.[3]

Although coronary occlusions in the nondiabetic occur several times more frequently in males than in females, diabetes obliterates this sex difference, in addition to increasing the over-all incidence.[4, 5] The severity, adequacy of control, and duration of diabetes influence the development of arteriosclerosis. Some investigators have presented evidence that poor control favors a high incidence of vascular disease,[6-8] but equally impressive reports show no correlation between the level of control and protection against vascular complications.[9, 10] Indeed, it might be anticipated that vascular lesions would develop in all diabetics if they survive for a sufficient period.[11] The duration of diabetes is an important predisposing factor to vascular disease at all ages,[6, 9, 12, 13] but it exerts its most profound effect in the younger age groups.[14] In regard to this last factor, White and Waskow have reported evidence of vascular disease in 92 per cent of a group of juvenile diabetics who lived 20 years or more following onset of the disease.[6]

The biochemical abnormalities in diabetes have been studied extensively with regard to their possible causal relationship to the genesis of arteriosclerosis. There seems to be no valid reason to implicate hyperglycemia per se as an etiological agent of arteriosclerosis in diabetics, but it has been suggested that wide fluctuations in blood sugar level may be harmful.[15] The presence of mucopolysaccharides in the ground substance and basement membranes of normal blood vessels raises the question of the importance of altered metabo-

lism of these substances in increasing the susceptibility to vascular disease.[16] McManus[17] has suggested that mucopolysaccharides might be deposited from the blood stream into renal lesions. Mucopolysaccharides indeed occur in both the retinal and glomerular lesions in the diabetic. According to Berkman and his co-workers,[18] diabetic patients with clinically evident vascular disease show higher levels of protein-bound polysaccharides and glucosamine in the serum than those without. These compounds have been found by others to be elevated in diabetic subjects regardless of the presence or apparent absence of vascular alterations.[19] The urinary excretion of acid mucopolysaccharides seems to be unrelated, however, to diabetes or its complications.[20] The higher levels of these substances in the blood may be the result rather than the cause of the renal and other degenerative changes in the diabetic.

The relationship of lipid levels to atherosclerosis has received considerable attention in both the diabetic and the nondiabetic. In a series of normal and well-controlled diabetic children, Chaikoff and his co-workers[21] found no differences in serum cholesterol, phospholipids, and fatty acids. In adult diabetics, hypercholesteremia occurs in the presence of hepatic and renal complications but, in uncomplicated cases, cholesterol levels are not unusually high. In each of two studies[22, 23] blood cholesterol levels were observed to be above normal in about one half of the diabetic patients. The S_f 12 to 20 fraction of lipoproteins in diabetics exhibits a tendency to be higher in the less well-controlled cases.[24] Available evidence, however, does not demonstrate consistent differences in this fraction between normal and diabetic persons.[25, 26] High lipid levels may be associated with the presence of severe atherosclerosis in diabetics but, as in nondiabetics, many severe cases of atherosclerosis may have normal lipid levels. In diabetics with retinal and, particularly, renal vascular changes, the S_f 12 to 20 lipoprotein levels are reported to be definitely elevated.[24, 27] The observations of Hirsch and his colleagues[28] point to a direct relation between hyperglycemia and hyperlipemia in the human diabetic and emphasize the importance of good control of the blood sugar level. These investigators showed that the developing hyperglycemia in diabetics following withdrawal of insulin therapy was associated with a parallel rise in esterified fatty acids of the blood. The lipid fraction returned to the normal range when the hyperglycemic diabetic subjects again became normoglycemic as a result of dietary and insulin control.

Ricketts[29] has pointed out the failure of evidence thus far available to indicate that the increased incidence of atherosclerosis in diabetes is related to a disturbance in lipid metabolism or to an elevation of any of the serum lipid fractions. Adlersberg and his co-workers[30] hold the opinion that the increases of serum lipid and carbohydrate components that have been observed may both be pathogenetically related in the development of diabetic retinopathy and diabetic glomerulosclerosis.

Because of the association in man between diabetes and arteriosclerosis, many experimental studies have been conducted in animals to determine the influence of the diabetic state on atherogenesis. It is important to note the marked species differences in susceptibility to arteriosclerosis and the divergent

opinions regarding the degree of similarity of experimental lesions to those found in man. Methods employed to induce pancreatic deficiency in animals include total or partial pancreatectomy, arterial and pancreatic duct ligation, toxic destruction of islet cells, and administration of anterior pituitary hormones and adrenal cortical hormones.

Dragstedt[31] observed a twelvefold greater incidence of arteriosclerosis in totally depancreatized dogs that were controlled with insulin and fed a relatively high-fat diet than in the control dogs. The lipid levels of the affected dogs were within the normal range or reduced. In a smaller series he noted that partially depancreatized dogs rarely showed vascular changes. Allen and Lisa[32] failed to find vascular alterations in a partially depancreatized dog with diabetes controlled for twelve years by insulin and diet. Insulin-maintained pancreatectomized dogs fed a diet low in animal fat did not develop atherosclerosis, according to Chaikoff and Kaplan.[33] The defects in lipid metabolism, notably hypolipemia and fatty liver, observable in depancreatized dogs given insulin, can be corrected by the addition of raw pancreas to the diet. Neither choline nor inositol, both known to be nutritional factors in pancreas, prevent the hypercholesteremia or hyperlipemia in cholesterol-fed chickens, nor do they reduce the incidence or severity of atherosclerotic lesions.[34]

Stamler and his co-workers[35] have determined the effect of pancreatectomy on various types of experimental atherosclerosis in the chicken. It should be pointed out here that removal of the pancreas in this species does not lead to sustained effects on blood sugar levels nor to altered glucose tolerance. Depancreatized cockerels on a normal diet showed no significant differences in plasma lipid patterns from intact birds, but those fed cottonseed oil in addition to cholesterol responded with a marked hypercholesteremia and a moderate hyperphospholipemia and showed severe cholesterol-induced atherosclerosis.[35] The addition of pancreatic factors to the mash of depancreatized, cholesterol-fed chicks was without influence on cholesterol levels or atherosclerosis.[36]

Renal glomerular lesions have been described in rats rendered diabetic by partial pancreatectomy[37] and in a dog made diabetic with anterior pituitary extract and maintained in the diabetic state for five years.[38] The relationship of these lesions to intercapillary glomerulosclerosis in diabetic humans is as yet unclear. The administration of cortisone to rabbits produces glomerular lesions that resemble the exudative type of human lesion.[39-41]

Two principal cell types, namely the alpha and beta cells, are found in the islets of Langerhans of the pancreas; these are differentiated by the selective staining of their constituent granules. Studies conducted even prior to the isolation of insulin from the pancreas by Banting and Best[42] indicated that the beta cells were the source of antidiabetic hormone.[43] More definitive evidence that the beta cell granules represent stored insulin or a precursor thereof is provided by relatively recent work.[44] Although certain reports[45, 46] suggest that the alpha cells are the site of glucagon, a hyperglycemic-glycogenolytic principle, this has not been unequivocally demonstrated.[47]

Selective damage of the insulin-producing beta cells in experimental animals

by alloxan has been shown to result in diabetes.[48-50] Attempts to induce atherosclerosis in alloxan diabetic rats by the feeding of cholesterol have been unsuccessful,[51] but it is well known that this species is unusually resistant to vascular sclerosis. Glomerular lesions, however, have been described by Mann and Goddard[52] in alloxan diabetic rats. Both renal and retinal lesions have been produced by the administration of cortisone in rabbits made diabetic with alloxan.[39] Protracted alloxan diabetes in rabbits does not lead to atherosclerosis.[53] Of considerable interest is the fact that the presence of alloxan diabetes in rabbits retards atherogenesis due to cholesterol feeding.[54] Treatment of such rabbits with insulin ameliorates the diabetic state and counteracts the inhibitory effect on atherogenesis due to the diabetes.[55] This inhibitory effect of alloxan diabetes on atherogenesis has been considered to be due to elevated levels of phospholipids and neutral fats accompanying the hypercholesteremia.[54] An alternate explanation is that in spite of very high levels of cholesterol its presence in lipoproteins of high S_f units seems to render it nonatherogenic.[56]

Alloxan does not produce atherosclerosis or even permanent diabetes in the guinea pig. It has been thought that the guinea pig is resistant to the diabetogenic action of this agent because of the ability of pancreatic islets to reform from ducts and acinar tissue.[57] A recent study seems to negate this presumption, since even animals with atrophic acinar tissue due to pancreatic duct ligation failed to become diabetic following alloxan administration.[58]

The injection of cobalt[59] or Synthalin A*[60] allegedly produces selective destruction of the alpha cells of pancreatic islet tissue. Neither complete nor permanent disappearance of the alpha cells occurs,[61] however, and the effect of these agents on the alpha cells therefore is not comparable to that produced by alloxan on the beta cells. The effect of cobaltous chloride injection on blood sugar level varies from a marked rise in rabbits[62] and dogs[63] to no significant change in the rat.[64] Fodden and Read[65] found that whereas Synthalin A abolished the extractable hyperglycemic-glycogenolytic factor of the pancreas of the rabbit, cobalt increased the amount of this factor in the pancreas. Caren and Carbo[66] observed a marked elevation of the plasma cholesterol level associated with the destruction of alpha cells in rabbits following intravenous administration of cobaltous chloride. These investigators interpreted their findings as suggesting that the alpha cells may secrete a hormone that regulates cholesterol metabolism. Evidence suggesting a diabetogenic action for glucagon has been presented.[67-69] Kenny[70] assayed human pancreas obtained at autopsy for extractable glucagon and found no significant differences between diabetics and nondiabetics. His results do not provide support for the view that glucagon plays a major role in the pathogenesis of diabetes mellitus. There appear to be no reports that the administration of glucagon or the stimulation or destruction of the alpha cells causes atherosclerosis.

Baló and Banga[71] have demonstrated in beef pancreas the presence of an elastolytic enzyme that they term elastase. Evidence has been reported that this enzyme is produced by the alpha cells,[72, 73] but a recent publication of

* Product of Schering Kahlbaum, East Berlin, Germany.

Cohen and his co-workers[74] points to the acinar cells as the source of elastase. A possible relationship of this enzyme to arterial disease was suggested by the finding that the pancreatic elastase content is diminished in atherosclerotic humans.[75] Experimental studies reveal, however, that whereas elastase retards the development of atherosclerosis in cholesterol-fed rabbits,[76] its oral or intramuscular administration to cholesterol-fed chickens fails to reduce either the incidence or severity of atherosclerosis.[77] Tennent *et al.*[77] also found that trypsin was without beneficial effect.

Original Investigations

Several experiments relating to the general problem of pancreatic function and arteriosclerosis were conducted by the author in association with other investigators. Studies on the effects of cobaltous chloride on cholesterol-induced atherosclerosis in chickens were done in collaboration with W. H. Ott and D. M. Tennent, and studies on arteriosclerosis in pyridoxine-deficient monkeys in collaboration with G. A. Emerson. Other publications will contain more detailed accounts of the cobalt studies[78] and of those on pyridoxine deficiency.[79]

Cobalt Study in Chickens

An examination of the influence of cobaltous chloride on atherosclerosis in chickens was prompted by reports that cobalt damages the pancreatic alpha cells of several species.[59, 61, 63] A recent publication, furthermore, shows that the injection of cobaltous chloride causes a moderate hyperglycemia in the chicken.[80]

White Leghorn cockerels, obtained at 1 or 2 days of age, were fed a basal ration[81] until they were 8 weeks of age. From the age of 8 to 16 weeks they received an atherogenic diet patterned after the basal ration, but 7 per cent of the corn meal was replaced with 2 per cent U.S.P. cholesterol and 5 per cent destearinated cottonseed oil. Throughout the experimental period the chickens were battery-confined and were permitted access to feed and water ad libitum. Ten birds were used per test group. Since preliminary experience had shown $CoCl_2 \cdot 6H_2O$ to be quite toxic when fed in the diet at 0.5 per cent for an 8-week period, it was given at this concentration for only 4 weeks (twelfth to sixteenth week of the test) in the present study. The chickens were sacrificed at 16 weeks of age. Atherosclerotic lesions in the thoracic aorta, abdominal aorta, iliac arteries, brachiocephalic arteries, and common carotid arteries were scored as described earlier.[81] The incidence of lesions affecting the valves of the heart was also noted. Weights were taken on liver, heart, testes, comb, and pancreas. The volume of bile in the gall bladder was measured, and the gizzard erosion score was recorded.[82]

The results obtained in 3 separate experiments are shown in TABLES 1 and 2. Birds receiving cobalt chloride consistently exhibited a lower incidence and severity of atherosclerosis in the major arterial vessels and a lower incidence of heart valve lesions. Cobalt chloride caused a moderate depression in body weight gain, an increase in relative weight of liver and heart, a striking de-

TABLE 1

BODY WEIGHTS, INCIDENCE AND SEVERITY OF ATHEROSCLEROSIS AND INCIDENCE OF VALVULAR LESIONS IN CHOLESTEROL-FED COCKERELS GIVEN COBALTOUS CHLORIDE IN THE DIET

Expt. No.	Treatment	Final No. birds	Final body weight	Atherosclerosis—per cent incidence (severity score)					Heart valves—per cent affected		
				Thoracic aorta	Abdominal aorta	Iliacs	Brachio-cephalics	Common carotids	Aortic	Mitral	Pulm.
1	Diet only	10	1730	92 (2.1)	60 (1.1)	90 (1.6)	100 (3.0)	80 (3.0)	90	90	30
	$CoCl_2$*	7	1010	86 (1.9)	43 (0.9)	29 (0.4)	86 (2.3)	57 (1.2)	57	29	17
2	Diet only	10	1740	60 (1.0)	40 (0.6)	50 (0.6)	60 (1.0)	50 (1.0)	80	50	30
	$CoCl_2$*	10	1170	30 (0.6)	40 (0.5)	40 (0.6)	40 (0.8)	40 (0.8)	40	10	20
3	Diet only	10	1710	100 (2.0)	70 (1.0)	70 (0.7)	100 (2.4)	60 (0.8)	80	60	0
	Diet only	10	1760	90 (2.4)	60 (1.1)	60 (0.9)	100 (2.7)	80 (1.2)	90	50	0
	$CoCl_2$*	10	1180	70 (1.3)	40 (0.6)	60 (0.6)	80 (1.7)	50 (0.7)	50	30	10
1, 2, 3	Diet only	40	1735	85 (1.9)	58 (1.3)	68 (0.9)	90 (2.3)	68 (1.5)	85	62	15
	$CoCl_2$*	27	1120	62 (1.3)	41 (0.7)	43 (0.5)	69 (1.6)	49 (0.9)	49	23	16

* $CoCl_2 \cdot 6H_2O$—0.5 per cent in diet during weeks 12 to 16.

TABLE 2

ORGAN WEIGHT: BODY WEIGHT RATIOS, BILE VOLUMES AND GIZZARD EROSION SCORES IN CHOLESTEROL-FED COCKERELS GIVEN COBALTOUS CHLORIDE IN THE DIET

Expt. No.	Treatment	Organ weight/body weight (percentages)					Vol. bile cc.	Gizzard erosion score
		Liver	Heart	Testes	Comb	Pancreas		
1	Diet only	2.39	0.503	0.180	2.35	0.188	1.6	0.6
	$CoCl_2$*	2.75	0.527	0.017	0.917	0.212	4.8	0.9
2	Diet only	2.12	0.421	0.520	2.73	0.182	2.2	0.4
	$CoCl_2$*	2.86	0.458	0.027	0.994	0.216	3.6	3.3
3	Diet only	2.33	0.408	0.234	2.96	0.187	3.0	0.1
	Diet only	2.22	0.406	0.347	2.89	0.192	1.7	0.2
	$CoCl_2$*	2.59	0.446	0.034	1.15	0.177	5.9	1.2
1, 2, 3	Diet only	2.26	0.434	0.320	2.73	0.187	2.1	0.3
	$CoCl_2$*	2.73	0.477	0.026	1.02	0.202	4.8	1.8

* $CoCl_2 \cdot 6H_2O$—0.5 per cent in diet during weeks 12 to 16.

crease in testis and comb weight, and an increase in bile volume and severity of gizzard erosions. The relative pancreas weights in the cobalt-treated and control groups were approximately equal.

The significance of the depressed growth rate due to cobalt chloride as a factor in inhibiting the development of atherosclerosis has been clarified in an extension of the present study. Thus it has been shown that atherosclerosis can be significantly decreased by the feeding of cobalt in the diet at concentrations too low to affect growth.[78] Furthermore, since the alpha cells of cobalt-treated chickens appeared undamaged on histological examination,[78] it seems unlikely that cobalt exerts its influence on atherosclerosis in this species through pancreatic changes. In view of the finding that ferric chloride[83] inhibits the absorption of cholesterol from the gut, the possibility exists that cobalt acts in a similar manner. The increased bile content of the gall bladders in cobalt-fed birds might be interpreted to suggest increased metabolism and excretion of cholesterol, but further studies on the composition of bile and on fecal excretion of cholesterol are required to clarify this possibility.

Pyridoxine Deficiency in Monkeys

The occurrence of arteriosclerosis in rhesus monkeys subjected to prolonged pyridoxine deficiency was first reported by Rinehart and Greenberg.[84] Using both the pyridoxine-deficient diet of these authors and a more completely purified and supplemented diet of their own, Mushett and Emerson[85, 86] were able to confirm and extend this observation. Because of the pancreatic changes that were seen in association with arteriosclerosis in our pyridoxine-deficient monkeys, some of the pertinent findings of this study are presented.

Arteriosclerotic lesions occurred regularly in both male and female rhesus monkeys fed a pyridoxine-deficient diet within 9 months and as early as 4½ months. The most common sites of the lesions were the lower abdominal aorta and the iliac arteries. Pale, slightly raised plaques were usually de-

tectable in these areas on gross inspection. In addition to the lesions of the larger vessels, the deficient animals exhibited arteriosclerosis in one or more of the following organs: heart, kidney, pancreas, testis, ovary, uterus, thymus, liver, adrenal, colon, and lung.

Microscopically, the arteriosclerotic lesion exhibits an increase in mucinous ground substance of the intima associated with cellular proliferation and deposition of collagen and elastic tissue fibers. Degeneration and duplication of the internal elastic lamella occur in some vessels. Swelling of the ground substance is seen infrequently in the media. No sudanophilic substance has been observed in the intimal plaques. Typical arteriosclerotic lesions in the abdominal aorta, kidney, and pancreas of the deficient animals are shown in FIGURES 1, 2, and 3.

Of particular interest in pyridoxine-deficient monkeys with arteriosclerosis is the presence of hyperplasia and hypertrophy of the pancreatic islets (FIGURE 4). As many as 25 individual islets have been observed in a single low-power microscopic field, and certain areas were noted to contain more islet tissue than acinar tissue. Some of the islets were quite large, the largest thus far

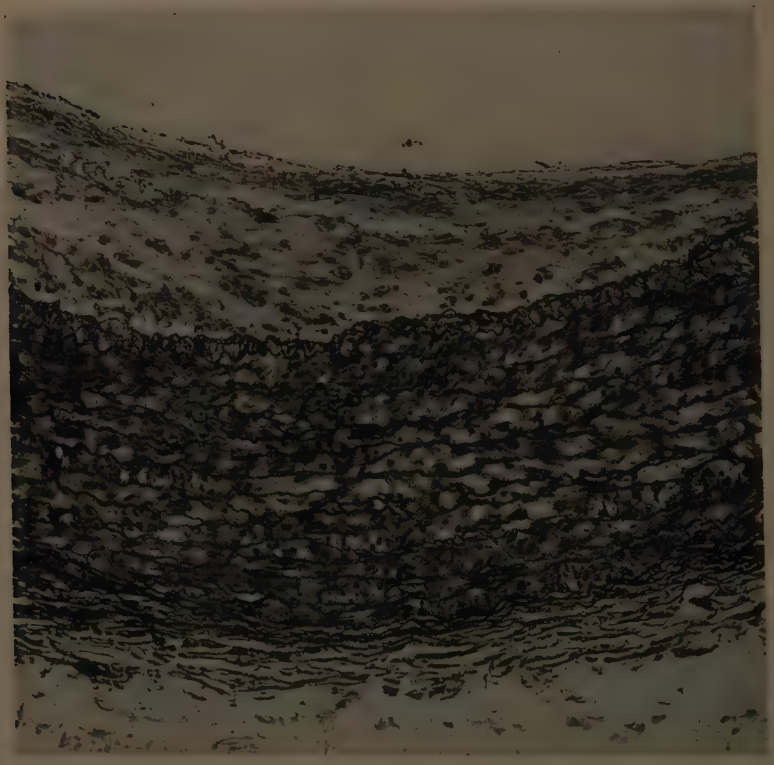

FIGURE 1. Abdominal aorta, showing cellular proliferation and increased ground substance in thickened intima and swelling of ground substance in media. Pyridoxine deficiency of 9 months. Hematoxylin-eosin stain. × 240.

observed having a length of 800μ (0.8 mm.), a macroscopically discernible dimension. Selective staining of the islets by several methods, including the aldehyde-fuchsin stain,[87] revealed an absolute increase in both alpha and beta cells. In some of the larger islets the alpha cells were increased relatively more in number than were the beta cells.

The pyridoxine-deficient monkeys also exhibited a relative enlargement of liver, kidneys, heart, thyroids, and pituitary. The adrenal glands showed an absolute as well as relative weight increase. In such enlarged adrenals, the inner zones of the cortex appeared widened, whereas the glomerulosa, on the average, was narrower than normal.

The close resemblance of arteriosclerotic lesions in pyridoxine-deficient monkeys to the early lesions of coronary arteriosclerosis in man has been pointed out.[88] The lack of correlation between lipid and early arteriosclerotic changes (increased ground substance, fibroblastic proliferation, and degeneration of elastic tissue) led Moon and Rinehart[89] to suggest that deposition of lipid is not the initiating factor in the development of human arteriosclerosis. The presence of excessive mucinous ground substance, which has a marked affinity for lipoidal material, especially cholesterol,[90] could reasonably account

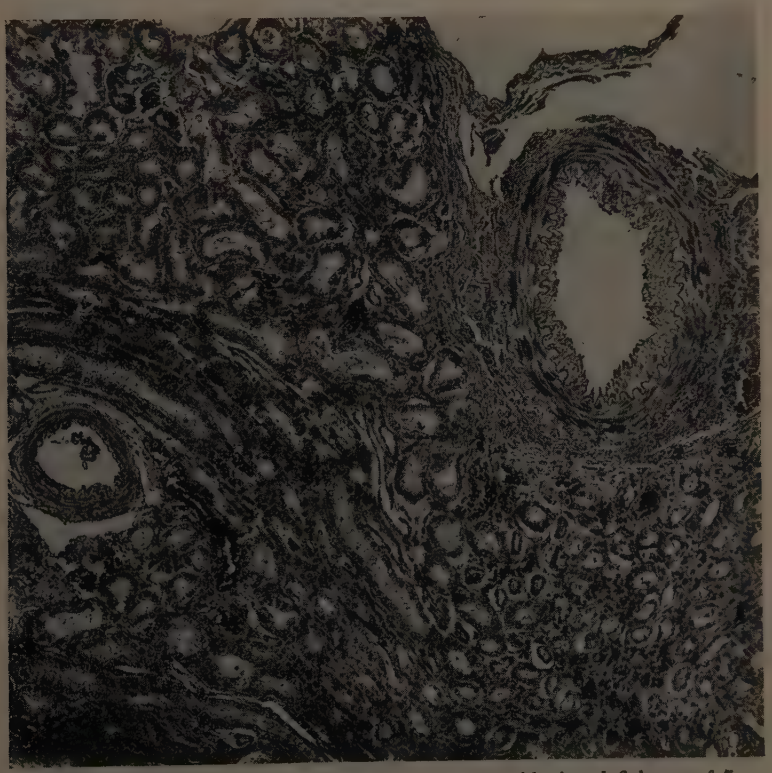

FIGURE 2. Renal arteries showing sclerotic intima. Pyridoxine deficiency of 7 months. Hematoxylin-eosin stain. × 240.

for the subsequent deposition of lipid as seen in the fully developed atheroma of man. It might be anticipated that lipid deposition would be favored by elevated plasma lipid levels, as well as by hypertension.

In view of the participation of pyridoxine in protein, amino acid, carbohydrate, and fat metabolism,[91-94] it is of more than passing interest that a lack of this vitamin leads, in the monkey, to alterations in the intimal ground substance, which is composed of mucopolysaccharide and mucoprotein components. Biochemical elucidation of the changes occurring in the ground substance in early arteriosclerosis, particularly in relation to the enzyme systems involved, should be rewarding. Snell has pointed out that pyridoxal phosphate, the coenzyme form of pyridoxine, participates in at least 20 enzymatic processes within the animal body and that these are not all affected to the same degree by pyridoxine lack.[95] It is not inconceivable, therefore, that a marginal or slightly suboptimal intake of the vitamin might result in subtle enzymatic defects without manifest clinical signs. It has been suggested that the usual diet in the United States may be marginal with respect to the estimated requirement of pyridoxine during certain seasons of the year.[96] Experimental studies in animals[97] and man[98] reveal that pyridoxine deficiency leads

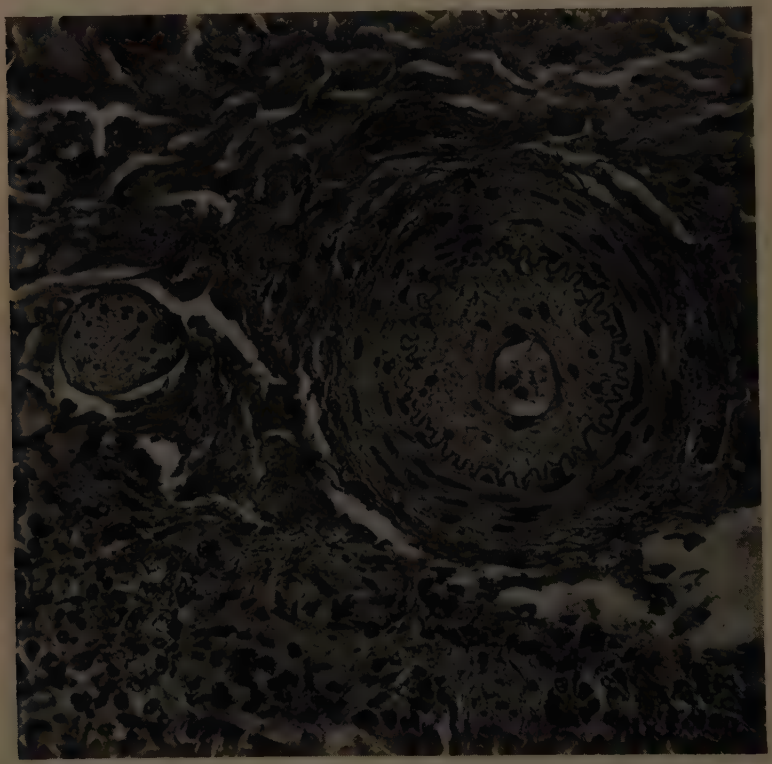

FIGURE 3. Pancreatic artery, showing marked occlusive intimal fibrosis. Pyridoxine deficiency of 9 months. Hematoxylin-eosin stain. × 240.

rather early to a defect in tryptophan metabolism characterized by excessive xanthurenic acid excretion. Diabetic patients, with and without retinopathy, have been shown to excrete more xanthurenic acid after a tryptophan test dose than do normal subjects; such xanthurenic acid excretion was greatly reduced after pyridoxine administration.[99] Similar findings in diabetic patients are reported in another communication.[100]

Pertinent to the present observation of adrenal cortical hyperplasia in arteriosclerotic pyridoxine-deficient monkeys is the relationship of adrenal cortical hyperplasia to several disorders in man recently reported by Moolten,[101] who noted a remarkably high association between adrenal cortical hyperplasia (or adenoma) and diabetes. Both nondiabetic and diabetic patients with adrenal hyperplasia had an increased incidence of coronary heart disease, and the incidence was greater in the diabetic group. Arterial hypertension, which is known to play an important role in arteriosclerosis, was recorded three times as often in patients with adrenal hyperplasia as in those without hyperplasia. Blood pressure determinations were not made in the pyridoxine-deficient monkeys described in the present report. In pyridoxine-deficient rats, however, significant arterial hypertension and adrenal gland enlargement have been reported, but arteriosclerosis was not observed in this species.[102]

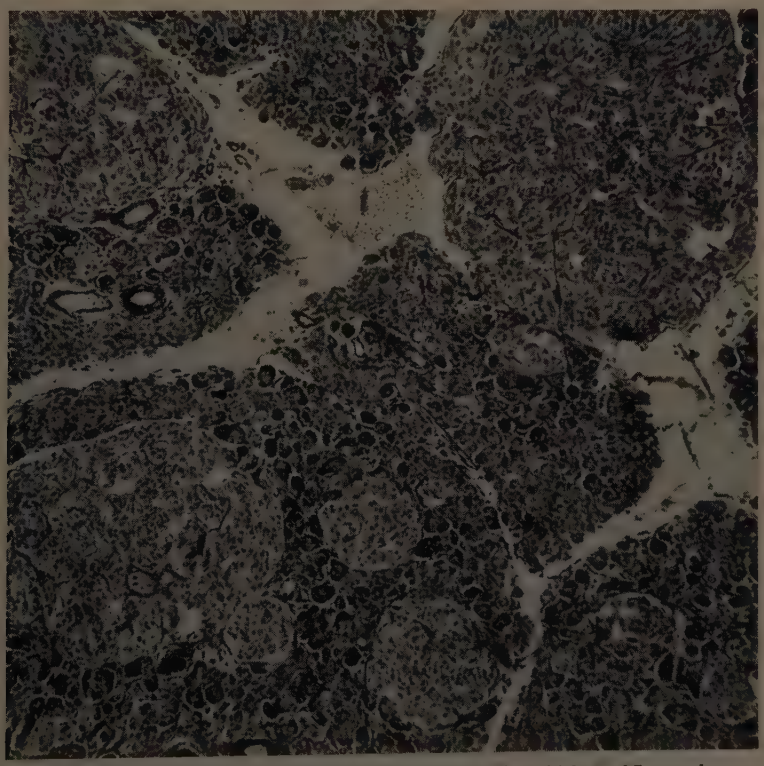

FIGURE 4. Pancreas, showing hyperplasia and hypertrophy of islets of Langerhans. Pyridoxine deficiency of 9½ months. Periodic acid-Schiff stain. × 240.

The relationship between increased absolute numbers of both alpha and beta cells and the presence of arteriosclerosis in pyridoxine-deficient monkeys remains to be clarified. Unfortunately, biochemical studies on carbohydrate metabolism, which might have revealed an imbalance of islet cell hormones, were not performed. Further exploration of this relationship may provide a better understanding of the nature and origin of the arteriosclerotic lesions observed in the pyridoxine-deficient animals and perhaps also of spontaneous arteriosclerosis in man.

Summary

A review of the literature pertinent to the role of the pancreas in the development of cardiovascular disease has been presented. The pancreatic disease, diabetes mellitus, is associated with an increased incidence and relatively early onset of arteriosclerosis, but studies to date have not revealed an adequate explanation for this association. Investigations in animals also suggest that the pancreas may in some manner be involved in the genesis of vascular lesions.

Experimental studies conducted by the author in collaboration with others show that dietary cobaltous chloride exerts an inhibitory effect upon atherogenesis in the cholesterol-fed chicken, and that the arteriosclerosis that develops in pyridoxine-deficient monkeys may be associated with hypertrophy and hyperplasia of the pancreatic islets.

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DISCUSSION OF PART III

Simon Rodbard (*Chronic Disease Research Institute, University of Buffalo, Buffalo, N. Y.*): The presentation of Lehr, showing endocrine effects in experimental arterial muscular necrosis, makes it clear that these vascular alterations differ markedly from those resulting from disturbances in lipid metabolism.

In the search for methods for the diagnosis of the tendency to coronary occlusion, the attempts to associate specific plasma lipid patterns with atherosclerosis are of the utmost importance. The lipid changes induced by administering various estrogens to men and women discussed in the paper of Robinson *et al.* indicate the wide variety of responses of the plasma lipoproteins to apparently similar hormonal agents. The plasma lipids become redistributed, depending on the physiological forces at work, as these investigators have shown.

So many factors participate in the atherosclerotic process that the solution of this key problem remains frustratingly elusive. Thus, the age period of the animal modifies the plasma lipid response to the ingestion of cholesterol.[1] Prior to puberty, cholesterol fed to the chick has only a minimal effect on the plasma level, and lipid infiltration of the aorta and the coronary arteries does not occur. In man, also, diets rich in fats and cholesterol such as occur in normal infancy produce no arterial disease.

At the onset of puberty a striking change occurs, not only in the plasma cholesterol concentration, but also in the resistance of the arteries to infiltration with lipids. In the pubertal chick the plasma cholesterol level increases, the blood vessels show an almost explosive infiltration with lipids, and atheromata develop in the aorta and in the coronaries within approximately a week. Similarly, in children with xanthomatosis and hypercholesteremia, coronary occlusion may occur at the onset of puberty. Possibly the arterial lesions develop very rapidly at this age because of the marked changes in hormonal patterns that assist in cholesterol regulation, as well as in a modified response of the blood vessels. The patterns continue to change during the period of adolescence. In animals, the large diffusely lipid-laden atheromata change within a few weeks of puberty to small, discrete atherosclerotic lesions with lipid only in the deeper layers. In man, given a normal regulation of cholesterol, the first atheromatous lesions begin to present themselves at this time of life.

Robinson *et al.* have told us of some of the effects of hormones on another critical age period, namely, the menopause. This period is critical in women since, at this stage of development, the incidence of coronary disease rises sharply. However, the data of these investigators suggest that the effects are due not so much to chronology as to the changes in hormonal pattern. My colleague Winkelstein has observed an increased incidence of coronary disease after the induction of artificial menopause.[2] The frequency of a history of abortion was also significantly higher in women with myocardial infarctions

than in controls, suggesting further the possible role of the sex hormones in protecting the coronary vessels. However, other factors play key roles in regulating lipid metabolism, since even large doses of estradiol were not adequate to reverse the lipid picture in women with grossly abnormal distributions.

The specificity of response of different arterial beds to plasma lipids also complicates the unraveling of the knotted skein of atherosclerosis. For example, the administration of estrogen in the chick raises the plasma cholesterol level and aggravates the lesions in the aorta; however, the coronary, cerebral, and renal arteries develop a remarkable protection against lipid infiltration and atheromatosis, even in the face of the hypercholesteremia.[3]

The metabolic activity of the vessel wall undoubtedly contributes to the protective process. After feeding cholesterol for one or two weeks, which is sufficient to produce acute hypercholesteremia and atherosclerosis, a return to a noncholesterol diet results in a fairly rapid elimination of lipids from the blood and in a progressive unloading of the lipids from the arterial wall. The administration of thyroid extract during the unloading period enhances somewhat the rate at which the cholesterol leaves the arteries. The presentation of Jones *et al.* on the role of the thyroid brings clarity to a long-confused problem. Despite the high concentrations of lipoproteins in myxedema, the beta-lipoproteins are lower in concentration than would be expected in coronary disease. Furthermore, the high cholesterol level of reduced thyroid function is not associated with an increase in coronary disease. These results provide some support for the work of Blumgart,[4] who long has advocated suppression of thyroid function in the treatment of angina pectoris. It may turn out that, in addition to reducing the effort required of the heart by lowering the metabolic rate, suppression of thyroid function may also cause changes in the lipoproteins and perhaps modify the vascular responses to the circulating lipids.

The blood pressure also participates in the tendency to atherosclerosis. The tendency to lipid infiltration is slight in the pulmonary arteries where the arterial pressure is normally low. When pulmonary arterial hypertension supervenes, however, atherosclerotic changes appear in the lesser circuit, as they do in systemic arteries where the pressure is high.

It is clear that the influence of hormones on lipid metabolism is being examined from many points of view. The importance of this problem certainly demands a continued and expanded effort to clarify within the shortest possible period the relations among cholesterol ingestion, the lipid concentrations in the blood, and the role of the endocrines on the atherosclerotic process.

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Part IV. Endocrines and Ground Substance

ENZYME ACTIVITIES OF HUMAN ARTERIAL TISSUE

John E. Kirk

Division of Gerontology, Washington University School of Medicine, St. Louis, Mo.

Most of the publications dealing with the enzyme systems of human arterial tissue are of recent date and, thus far, many aspects of this subject have not been investigated. Consequently, the importance of the metabolism of the arterial wall in the pathogenesis of arteriosclerosis remains uncertain. In view of the significance of arteriosclerosis as a disease entity, the initiation of systematic studies on arterial metabolism seems justified. Such investigations will make it possible to establish the general metabolic pattern of the arterial tissue and to demonstrate differences in enzymatic activities between normal and pathological tissue areas. A specific aim of studies on arterial tissue is the identification of the limiting enzyme systems within the two main metabolic subdivisions: (1) the glycolytic pathway and (2) the tricarboxylic acid cycle. After the limiting enzymes in these pathways have been established through studies on normal arterial tissue it will be of considerable interest to ascertain whether the activities of these particular enzymes are affected by the presence of arteriosclerosis. This is one of the areas in which enzyme studies on arterial tissue may provide information of direct or indirect value for the conception of the pathogenesis of arteriosclerosis. It is realized that even when such data have been furnished it may be difficult to determine whether observed metabolic changes of the tissue are the cause or the result of arteriosclerosis. In spite of these interpretative limitations the systematic collection of data on the enzyme activities of normal and arteriosclerotic tissue constitutes a necessary approach to the subject.

The procedures used to ascertain the presence and activity rates of enzymes in a tissue include chemical and histochemical techniques. Although a loss in enzyme activity frequently accompanies the steps associated with the histological preparatory procedures, such tissue studies often give valuable information about the localization of the enzymes in the tissue. In the present review, which is limited to observations on human arterial tissue, the results of both types of studies are considered.

The human aortic tissue is characterized by having a rather low respiratory rate, the Q_{O_2} of intact intima-media tissue sections of samples obtained shortly after death being about 0.30.[1] An interesting feature of the arterial metabolism is that the rate of glycolysis is comparatively high, the $Q_g^{O_2}$ value being close to 1.00.[1] The glycolysis rate of the human aortic tissue is relatively independent of the oxygen tension; in comparative studies on a series of aortic samples the average anaerobic glycolysis rate was found to be only about 30 per cent higher than the aerobic rate of glycolysis.

As a result of the high glycolysis rate of the tissue it has been calculated that the glycolysis accounts for about 50 per cent of the total energy production

by the human aorta; in this respect the arterial tissue can be placed in the same metabolic class as the lens of the eye.

Another notable aspect of the arterial metabolism is the fact that when aortic tissue is stored at 4° C. under sterile conditions both the respiratory and glycolytic capacities of the tissue are maintained for several weeks, although at definitely lower rates than the initially recorded values.[1]

The enzyme studies performed in my laboratory have been carried out on homogenates of arterial tissue obtained fresh at autopsy from individuals of various ages. The measurements have been conducted on intima-media samples of the aorta and pulmonary artery and, in many instances, also on coronary artery tissue. In the case of several enzymes a sufficient number of samples (from 60 to 100) has been included in the investigations to permit an evaluation of the relation of enzyme activity to age. In recent years the determinations have been made separately on normal and arteriosclerotic areas of the same blood vessels.

The procedures used for activity measurements have been of the conventional type, with such modifications as were necessitated by the characteristics of the arterial tissue. It is generally agreed that determinations of enzyme activities through the use of tissue homogenates with the employment of specific substrates supplemented by the necessary cofactors afford a satisfactory measurement of the quantities of various enzymes in the tissue and provide a useful basis for comparison of such values. It must be realized, however, that values determined in this way do not necessarily correspond to the activity values in the intact tissue, in which the concentrations of substrates and cofactors usually are lower, and where special regulatory mechanisms may be operating.

The studies completed or currently being carried out in my laboratory include several of the enzymes of the glycolytic and oxidative pathways, various phosphatases, and some specific enzymes. In addition, the tissue concentrations of some coenzymes have been determined.

Glycolytic and oxidative enzymes. The mean activities of some glycolytic and oxidative enzymes in homogenates prepared from human aortic, pulmonary artery, and coronary artery tissue are listed in TABLE 1. In addition to these enzymes, studies are in progress on the activities of enolase, TPN-isocitric dehydrogenase, and TPN-"malic enzyme" in arterial homogenates.

It will be noted from the values recorded in TABLE 1 that the enzyme activities of the aorta and pulmonary artery in general are of the same order of magnitude. In contrast to this, a comparison of the lactic and malic dehydrogenase values observed for the aorta and coronary artery shows that there exists a significant difference between the activities of these enzymes in the two arteries, the ratio lactic dehydrogenase/malic dehydrogenase being 1.94 for the aorta, but only 0.77 for the coronary artery.[3] These findings emphasize the fact that observations made on one type of artery do not necessarily apply directly to other blood vessels.

It will be seen further from TABLE 1 that our studies have demonstrated the presence of notable activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in the tissue.[6] This observation indicates

TABLE 1

MEAN ACTIVITIES OF GLYCOLYTIC AND OXIDATIVE ENZYMES OF HOMOGENATES OF HUMAN AORTIC, PULMONARY ARTERY, AND CORONARY ARTERY TISSUE

Values Represent Millimoles of Substrate Metabolized/Gram of Wet Tissue/Hour

Enzyme	Aorta	Pulmonary artery	Coronary artery	References
Glycolytic pathway				
Hexokinase	0.010	0.010		2
Phosphoglucoisomerase	1.620	1.510	1.820	2, 3
Aldolase	0.063	0.070	0.076	4
Lactic dehydrogenase	1.020	0.880	0.670	3, 5
Direct oxidative shunt				
Glucose-6-phosphate dehydrogenase*	0.108	0.081	0.089	6
6-Phosphogluconate dehydrogenase	0.011	0.015	0.021	6
Tricarboxylic acid cycle				
Aconitase	0.021			7
Fumarase	0.140	0.120		8
Malic dehydrogenase	0.530	0.440	0.870	5

* Values represent millimoles of triphosphopyridine nucleotide (TPN) reduced per gram of wet tissue per hour, using glucose-6-phosphate as substrate.

the functioning in the tissue of the direct oxidative shunt, a finding that is of definite interest since the existence of such a shunt presumably provides an alternate pathway for the metabolism of carbohydrates in the arterial tissue.

In the case of the aorta, the pulmonary artery, and the coronary artery the data recorded in the table reveal a marked difference in the quantities of substrate metabolized by the various enzymes within the glycolytic and oxidative cycles and the direct oxidative shunt. As an example, the average activity of hexokinase was found to be less than 1 per cent of the mean activity of phosphoglucoisomerase.[2] Similarly, the activity of 6-phosphogluconate dehydrogenase was, on the average, only 10 to 20 per cent of that of glucose-6-phosphate dehydrogenase.[6] These findings support the idea that investigations aimed at identifying the limiting enzyme systems may prove rewarding.

With regard to other oxidative enzymes the presence in the human aorta of succinic dehydrogenase has been established in investigations on intact tissue, both through the use of manometric procedures[9, 10] and by a quantitative tetrazolium technique.[11] Manometric studies on the cytochrome *c* oxidase activity of the tissue have further been reported by Maier and Haimovici.[10]

Phosphatases. In TABLE 2 the activities of various phosphatases in arterial homogenates have been recorded. The presence in human arterial tissue of a nonspecific phosphomonoesterase with a *p*H optimum at 5.7 was first reported by my laboratory in 1949.[16, 17] The previously assumed absence of such phosphatase in the arterial wall had for many years been a subject of importance in the discussion of the pathogenesis of arterial calcification. It was shown that failure to demonstrate the presence of this enzyme in the tissue could be ascribed to the fact that earlier phosphatase studies had been conducted only at an alkaline reaction.

A remarkable feature of both elastic and muscular arteries is the high activities of two specific phosphatases, adenosinetriphosphatase and 5′-nucleotidase

TABLE 2

MEAN ACTIVITIES OF VARIOUS PHOSPHATASES IN HOMOGENATES OF HUMAN ARTERIAL TISSUE
Values Represent Millimoles of PO_4 Liberated/Gram of Wet Tissue/Hour

Enzyme	*p*H	Aorta	Pulmonary artery	Coronary artery	References
Nonspecific phosphomonoesterase*	5.7	0.041	0.032	0.032	31
Adenosinetriphosphatase	7.0	0.056			12
Adenosinetriphosphatase	8.1	0.206	0.249	0.432	31
Adenosinetriphosphatase	9.0	0.117			12
Adenosinetriphosphatase	9.5	0.103			13
Inorganic pyrophosphatase	7.2	0.163	0.200	0.338	31
5′-Nucleotidase	7.5	0.110			14
5′-Nucleotidase	7.5	0.084			15
5′-Nucleotidase	7.5	0.123			13
5′-Nucleotidase	9.5	0.045			13

* Determinations made of millimoles paranitrophenol liberated per gram wet tissue per hour, using paranitrophenylphosphate as substrate.

in the tissue. Studies by Banga and Nowotny[12] indicate that human arterial tissue contains two different adenosinetriphosphatases, both of which are magnesium-activated. One of the phosphatases is believed to be bound to myosin-actomyosin, is water-insoluble, and has a *p*H optimum at 7.0; the other phosphatase is water-soluble and has an optimal activity at *p*H 9.0. The latter enzyme has been found only in arterial tissue and was designated aorta-adenylpyrophosphatase by Baló *et al.*[18] As might be expected, the muscular-walled arteries, like the femoral artery, were found to contain greater quantities of the myosin-bound enzyme than the aorta. In both muscular and elastic arteries, however, the special arterial adenosinetriphosphatase was present in the highest concentration.

Chemical and histological studies of the adenosinetriphosphatase activity of human aortic, coronary artery, and peripheral artery tissue have been reported further by Antonini and Weber,[13] who observed a higher activity of the enzyme at *p*H 9.5 than at 7.5.

In our investigations on the adenosinetriphosphatase activity of arterial tissue the measurements have been carried out at *p*H 8.1 in the presence of an optimal concentration of magnesium. In addition, determinations have been performed at *p*H 7.2 of the inorganic pyrophosphatase activities of the same homogenates. In the latter analyses sodium pyrophosphate instead of adenosinetriphosphate was used as substrate. The findings,[31] based on investigations of 86 samples, show that the activities of the two enzymes in the arterial tissue are approximately equal (TABLE 2). The adenosinetriphosphatase values observed in our studies are about twice as high as those reported by Banga and Nowotny[12] and by Antonini and Weber;[13] the data of these authors, however, were derived from only a small series of samples.

Although the exact nature of the enzyme(s) responsible for the adenosinetriphosphatase activity of arterial tissue has not yet been established, the presence in the tissue of this energy-producing mechanism deserves attention, particularly because the activity observed for the arterial tissue is of similar

magnitude as that found in striated muscles.[12] In view of the low oxidative metabolism of human arteries, the metabolic significance of the high adenosinetriphosphatase content of the tissue requires further consideration.

It will be seen from the data listed in TABLE 2 that the adenosinetriphosphatase activity of the coronary artery is higher than the activity found in the aorta and the pulmonary artery. It is likely that this difference is due to the greater amount of smooth muscle present in the wall of the coronary artery. Some interest has recently been attached to the adenosinetriphosphatase of the coronary arteries with the purpose of investigating the effect of vasodilating drugs on the activity of the enzyme, but only studies performed on adenosinetriphosphatase of coronary arteries of animals have thus far been reported.[19-21]

The presence in human arterial tissue of another specific phosphatase, 5′-nucleotidase, was first reported by Reis[14, 15] in 1950. This enzyme, which has a *p*H optimum at 7.8, acts only on adenosine-5-monophosphate and inosine-5-monophosphate. The activity of this enzyme in aortic tissue is unusual, since it has been found to be of the same order of magnitude as observed in ossifying cartilage and about 15 times higher than the activity in skeletal muscle tissue. The findings by Reis have been confirmed by Newman and his associates[22] and by Antonini and Weber.[13] It has been suggested by Reis that the high activity of this phosphatase at a physiological *p*H may be of importance for the process of tissue calcification.

Other enzymes. Of other enzymes studied in human arterial tissue, carbonic anhydrase is of special interest, since this enzyme may be indirectly associated with both the glycolytic and oxidative metabolism of the tissue. Investigations carried out on homogenates prepared from the media of human aortic samples showed an appreciable activity of the enzyme in all the samples studied;[23] the measured activity values were corrected for the minute amounts of red blood cells present in the homogenates. The mean enzyme value found corresponded to 0.01 enzyme units (E.U.)/mg. wet tissue[24] (TABLE 3). This enzyme concentration is only about 1 per cent of that found in the red blood cells, but is nevertheless noteworthy. Whether the significant function of the enzyme in the arterial tissue is a catalysis of the hydration of carbon dioxide or

TABLE 3

MEAN ACTIVITIES OF CARBONIC ANHYDRASE, BETA-GLUCURONIDASE, AND PHENOLSULFATASE IN HOMOGENATES OF HUMAN ARTERIAL TISSUE

Enzyme	Aorta	Pulmonary artery	Coronary artery	Unit	References
Carbonic anhydrase...	0.01			E.U.*/mg. wet tissue	23
Beta-glucuronidase....	0.22	0.25	0.17†	Micromoles phenolphthalein liberated/gram wet tissue/hour	25
Phenolsulfatase.....	0.015	0.020	0.018†	Micromoles *p*-nitrophenol liberated/gram wet tissue/hour	26

* Enzyme unit value based on colorimetric activity determination.[24]

† Values refer to individuals aged 3 to 39 years.

of the dehydration of carbonic acid is not known. If, however, carbonic acid is formed in large amounts in the arterial wall as a metabolic end product or through neutralization of lactic acid, a catalysis of the dehydration of carbonic acid might serve a useful purpose by facilitating the removal of the compound from the tissue, since the diffusion rate of carbon dioxide is higher than that of carbonic acid.

As seen from the data listed in TABLE 3, further investigations have been carried out on two special enzymes, beta-glucuronidase and phenolsulfatase.[25, 26] The roles of these enzymes in the metabolism of arterial tissue have not been established. The glucuronidase activity found in vascular tissue is about 50 to 100 times higher than the values reported for human plasma, but is rather low compared to the activity of the enzyme in parenchymatous organs. With regard to phenolsulfatase, sufficient observations on the activity of this enzyme in other human tissues are not available to permit comparisons.

Coenzymes. Studies on the coenzyme concentrations of human aortic tissue[27, 28] have shown comparatively high values for both nicotinic acid and riboflavin (TABLE 4). Since arterial tissue contains an active DPNase,[27] the determination of the nicotinic acid content of the tissue affords a more reliable measurement of the phosphopyridine nucleotide level than the direct analysis of the DPN tissue content. The nicotinic acid concentration recorded in TABLE 4 is about 20 per cent, and the total riboflavin value 10 per cent, of the corresponding values reported for human liver tissue.

For several years we have been engaged in investigations on the cytochrome *c* content of aortic tissue and have succeeded in isolating this compound from the tissue through a modification of Carruthers'[29] procedure. Because of the low cytochrome *c* concentration in the human aorta, exact determinations of the cytochrome *c* level in the tissue have not yet been possible.

Correlation between age and enzyme activities. The relation of enzyme activities and coenzyme concentrations of aortic tissue to the age of the subjects from whom the samples were obtained has been studied in some detail in our laboratory. If the values for children are excluded, an analysis of the data shows that the activities of several enzymes, namely hexokinase,[2] phosphoglucoisomerase,[2] malic dehydrogenase,[5] glucose-6-phosphate dehydrogenase,[6] 6-phosphogluconate dehydrogenase, the nonspecific phosphomonoesterase,[31] adenosinetriphosphatase,[31] and inorganic pyrophosphatase[31] remain essentially unchanged with age, whereas the activities of some other enzymes, fumarase[8]

TABLE 4

MEAN COENZYME CONCENTRATIONS OF HUMAN AORTIC TISSUE

Coenzyme	Micrograms/gram wet tissue	References
Nicotinic acid	19.2	27
Free riboflavin + flavin mononucleotide	0.40	28
Flavin adenine dinucleotide	0.76	28
Total riboflavin	1.16	28

and phenolsulfatase,[26] show a significant tendency to decrease with age. A definite reduction in activity with age was also noted by Maier and Haimovici[10] for succinic dehydrogenase and cytochrome oxidase of intact human aortic tissue.

In the case of three of the enzymes studied, aldolase,[4] lactic dehydrogenase,[5] and beta-glucuronidase,[25] the activities were found to increase until about 50 to 65 years; in the case of the two latter enzymes this increase was followed by a decrease in subsequent decades. A similar behavior has been found for some enzyme systems of other organs, as recently reviewed by Barrows.[30]

With regard to the coenzymes studied, the concentrations of both nicotinic acid and riboflavin in aortic tissue were found to decrease significantly with age.[27, 28] The coefficients of correlation between age and nicotinic acid concentration of the tissue and between age and total riboflavin concentration were, respectively, -0.37 ($t = 3.17$, $N = 61$) and -0.39 ($t = 4.20$, $N = 100$).

Arteriosclerotic tissue. As already mentioned, special investigations have been made of the enzyme activities of arteriosclerotic areas of aortic and coronary artery samples as compared with normal arterial tissue.

In TABLES 5 and 6 the mean values observed for arteriosclerotic aortic tissue have been listed in percentages of the values found for the normal tissue of the same samples. The values have been recorded for 3 age groups: 40 to 49 years, 50 to 59 years, and 60 to 75 years. To permit a more accurate evaluation of the enzyme changes in the arteriosclerotic tissue the activity values have been expressed both on the basis of wet tissue weight and tissue nitrogen content. Considerable differences in the enzyme activities of arteriosclerotic tissue specimens were often noted between individual samples within the same age groups, and it is possible that a regrouping of the data on the basis of the type of arteriosclerosis present in the tissue might yield additional information of value.

TABLE 5

ENZYME ACTIVITIES OF ARTERIOSCLEROTIC AORTIC TISSUE IN PERCENTAGES OF ACTIVITIES OF NORMAL ARTERIAL TISSUE OF SAME SAMPLES

Values Are Expressed on the Basis of Wet Tissue Weight

Enzyme	Age group			References
	40 to 49 years	50 to 59 years	60 to 75 years	
Hexokinase	92	87	76	2
Phosphoglucoisomerase	100	80	82	2
Lactic dehydrogenase	91	84	72	5
Glucose-6-phosphate dehydrogenase	65	68	65	6
6-Phosphogluconate dehydrogenase	126	94	94	6
Malic dehydrogenase	86	87	81	5
Nonspecific phosphomonoesterase	93	100	93	31
Adenosinetriphosphatase	94	80	78	31
Inorganic pyrophosphatase	89	85	83	31

TABLE 6

ENZYME ACTIVITIES OF ARTERIOSCLEROTIC AORTIC TISSUE IN PERCENTAGES OF ACTIVITIES OF NORMAL ARTERIAL TISSUE OF THE SAME SAMPLES
Values Are Expressed on the Basis of Tissue Nitrogen Content

Enzyme	Age group			References
	40 to 49 years	50 to 59 years	60 to 75 years	
Hexokinase	102	102	93	2
Phosphoglucoisomerase	111	96	95	2
Lactic dehydrogenase	97	95	93	5
Glucose-6-phosphate dehydrogenase	68	72	71	6
6-Phosphogluconate dehydrogenase	139	99	94	6
Malic dehydrogenase	94	100	108	5
Nonspecific phosphomonoesterase	94	110	95	31
Adenosinetriphosphatase	96	85	76	31
Inorganic pyrophosphatase	91	90	88	31

The data presented in TABLES 5 and 6 show that the various enzymes in the human aorta are affected to a different extent by the presence of arteriosclerotic tissue changes. During the earlier stages of arteriosclerosis, as represented by the samples from the 40- to 49-year-old subjects, many of the enzymes show no change in activity or only a moderate reduction. In contrast to this, the activity of one enzyme, namely, glucose-6-phosphate dehydrogenase,[6] was found to be significantly reduced in the arteriosclerotic samples from this age group.

The enzyme activities of the arteriosclerotic tissue specimens from the 60- to 75-year-old individuals, with the exception of 6-phosphogluconate dehydrogenase[6] and nonspecific phosphomonoesterase,[31] all show a definite decrease when calculated on the basis of wet tissue weight. In the case of several of the enzymes, namely, hexokinase,[2] phosphoglucoisomerase,[2] lactic dehydrogenase,[3] malic dehydrogenase,[3] and inorganic pyrophosphatase,[31] the decrease in activity is not statistically significant when the values are calculated on the basis of the tissue nitrogen content. It is of interest to note that the mean activities of the arteriosclerotic tissue samples are essentially the same for the 50- to 59- and 60- to 75-year groups.

Comparisons of enzyme activities of normal and arteriosclerotic human aortic tissue have also been reported by Banga and Nowotny[32] and by Antonini and Weber.[13] In the studies by Banga and Nowotny the average adenosinetriphosphatase activity of arteriosclerotic tissue, as measured at *p*H 7.0, was found to be 55 per cent of that of the normal aortic tissue; at *p*H 9.0 the mean activity was 76 per cent of that observed for the normal tissue.[32]

The investigations by Antonini and Weber included measurements of both the adenosinetriphosphatase (*p*H 9.5) and 5′-nucleotidase activities of the samples.[13] In addition, concomitant histological studies were performed. The observed reduction of the adenosinetriphosphatase activity of the arterio-

sclerotic samples averaged 23 per cent, and the reduction in 5′-nucleotidase activity, 21 per cent. The histological studies revealed that the reduction in the activities of these enzymes seemed to proceed in a manner parallel with a decrease in the number of muscle cells in the arterial wall during the development of arteriosclerosis. In peripheral arteries with Mönckeberg's sclerosis the adenosinetriphosphatase and 5′-nucleotidase activities were found to become negative in the muscle cells adjacent to the areas where the cellular structures were no longer distinguishable.

With regard to the coronary artery, a study of the lactic dehydrogenase, malic dehydrogenase, and phosphoglucoisomerase activities of normal and arteriosclerotic tissue of this artery was published recently from this laboratory.[3] The average activities of these enzymes found in the arteriosclerotic tissue were, respectively, 83, 67, and 77 per cent of those observed from the normal tissue when expressed on the basis of wet tissue weight. Similar investigations on other enzymes are in progress, but enzyme measurements on the coronary artery are somewhat limited by the comparatively small amounts of tissue available.

In conclusion, it may be stated that although the enzyme studies on human arterial tissue are as yet incomplete some findings seem to be of definite interest. Among these is the establishment of the presence in the arterial wall of an adenosinetriphosphatase activity that is of the same order of magnitude as that found in striated muscle tissue. This observation does not fit into the pattern that might be expected for a tissue with a low respiratory rate, and invites further considerations with regard to the character of the arterial metabolism. Another significant finding is the demonstration of an activity of 5′-nucleotidase in aortic tissue at a level similar to that found in ossifying cartilage. A detailed study of the possible function of this enzyme in the process of arterial calcification, as suggested by Reis,[14, 15] would seem advisable.

The investigations that have been performed thus far on human arteriosclerotic tissue have shown that the various enzymes are affected to a different degree by the arteriosclerotic changes. When these studies have been extended to include most of the enzymes usually present in mammalian tissues, a certain metabolic pattern for the arteriosclerotic tissue may emerge. If this should prove to be the case, the investigations may possibly provide some answers to the many-sided problem of the pathogenesis of arteriosclerosis.

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THE EFFECTS OF TISSUE AGE AND SEX UPON CONNECTIVE TISSUE METABOLISM

Robert J. Boucek, Nancy L. Noble, J. Frederick Woessner, Jr.
Howard Hughes Medical Institute, Miami, Fla.

The process of aging in man appears to be the result of at least two events in the tissue: the first or primary event consists of cellular alterations, while the secondary is the consequence of ischemia. Even though cellular aging is a primary phenomenon, clinical means are not available for its detection. In degenerative diseases, which are recognizable after the secondary aging phenomena have occurred, the primary event is an alteration of the arterial tree.

The vascular changes that the morphologist relates to aging are thickening of the intima and fraying of the internal elastic lamella.[1] The physical chemist recognizes vascular aging from the reduction of tissue elasticity.[2] The clinician diagnoses vascular aging by palpation of firm, tortuous and, at times, nonpulsating peripheral arteries, or by the observable effects of occlusive arterial phenomena, such as coronary, cerebral, renal, or femoral thrombosis. The occlusive arterial episode is usually due to a thrombus that overlies an intimal prominence, that is, an atheroma. The lipid nature of the atheroma has suggested to the clinical investigator a relationship, as yet elusive, between the serum lipids and atheromatosis. Pathologists do not agree on the order or on the nature of the events preceding the atheroma formation. It is thought by some that a fibrin deposit is laid down upon the endothelium and is subsequently replaced by fibrous connective tissue.[3] Others note that the primary event in the aging of the artery begins early in life and consists of intimal proliferation and collagen invasion into the zone of the internal elastic lamella and into the media.[4] All agree, however, that the changes in the intima are not uniform, but appear to be accentuated in certain areas where presumably a hemodynamic stress is developed during systole. Pathologists are familiar with the problem of a relatively young man with a fatal coronary thrombosis resulting from a discrete lesion in the left coronary artery, with the remainder of the coronary arteries quite patent and without excessive aging changes.

Irrespective of the approach to the understanding of the nature of vascular aging, two findings are outstanding: first, an alteration in the connective tissue of the vessel, and second, the marked male dominance in the incidence of early vascular changes. The connective tissue changes consist of an increase in the amount of collagen in the intima, the internal elastic lamella, and the media, and of a fraying and duplication of the internal elastic lamella. The addition of collagen to the intima and the interlacing of collagen between the elastic fibers of the internal elastic tunic would be expected to increase the rigidity of the vessel and thus to enhance the turbulence of the blood as it passes some angled portion of the vascular system. Localized turbulence causes an intimal reaction recognized as fibrogenesis by the morphologists and as the nidus of the atheroma by pathologists.

Thus, the biology and biochemistry of collagen occupy a principal area for the investigation and elucidation of the aging process. Collagen, a fibrous

protein that has been studied extensively by the protein chemists, the physical chemists, the leather chemists, the biologists, and the clinicians interested in rheumatic diseases, now is receiving increasing attention by investigators interested in the aging process. Collagen, which is derived from fibroblasts, begins as an intracellular polypeptide, possibly formed in the cisternae described by Porter,[5] and later appears extracellularly as a slender filament. The slender filaments become associated with each other and form a unit fiber with its electronmicrographic characteristic of 640 Å striae.[6] Other macromolecules of collagen are added to the unit fiber with age and thus cause an increase in its diameter.[7]

The leather chemists, as well as the biochemists, have noted two unusual amino acids in the polypeptide strands of collagen that are not essential in the dietary sense, but at least one of which is apparently required for collagen formation. These amino acids are hydroxyproline and hydroxylysine. Later, the physical chemists recognized the key relationship between hydroxyproline and the stability of the collagen fiber.[8] Since these hydroxylated amino acids are derived from either proline or lysine, the incorporation of amino acid into the polypeptide strand of the protofibril filament and its metabolism have been investigated with the isotopically labeled "parent" amino acids.[9] Apparently, oxidation of a portion of these amino acids is required for the formation of the protofibril. Failure to form hydroxyproline, for example, in the scorbutic guinea pig, results in the absence of identifiable collagen.[10] Both of these hydroxy amino acids are characteristic of collagen and constitute biochemical markers that are as distinctive to the chemist as the cross-striations of the fiber are to the morphologist. As C^{14}-labeled lysine became available, the system of the hydroxylation of lysine to hydroxylysine offered a means by which the rate of collagen synthesis might be followed.

Methods

Connective tissue was isolated from adult male and female Sprague-Dawley rats (6 to 18 months of age) by the technique described previously.[11] Two or three small squares of sterile Ivalon* sponge, which had been dried and individually weighed, were implanted subcutaneously, and the sponge connective tissues were removed from the animals after the desired period of tissue growth.

Tissue Preparation and Analyses

Immediately after removal, the sponge tissue was frozen and the surrounding fascia trimmed. The tissue was minced with scissors into 0.85 per cent saline (*p*H 7.4) and homogenized in a VirTis Macro Homogenizer† at a setting of 50 for 5 min. The homogenate was centrifuged at 600 g for 15 min. and the supernatant discarded. All procedures were performed at 0 to 5° C. The collagen of the saline-insoluble residue was extracted with hot (90° C.) trichloroacetic acid (TCA) by the method of Fitch *et al.*[12] The TCA extract was dried and subsequently hydrolyzed for 16 hours in 6 *N* HCl at 110° C. in a

* Produced by the Clay-Adams Co., Inc., New York, N. Y.
† Product of The VirTis Company Inc., Yonkers, N. Y.

sealed tube. An aliquot of the hydrolyzate was taken for nitrogen determination by the Conway technique[13] after Kjeldahl digestion. A second portion of the hydrolyzate was used for hydroxyproline determination[14] and column chromatography after it had been dried 3 times to remove HCl and neutralized to *p*H 7.0.

The basic amino acids of the TCA hydrolyzate were separated by elution from a Dowex-50 × 8 (200 to 400 mesh) column by the procedure of Moore and Stein.[15] Citrate buffer (*p*H 5.0) was used to elute the neutral and acidic acids, and phosphate buffer (*p*H 6.8) for the 3 basic amino acids, histidine, hydroxylysine, and lysine. Two-ml. fractions of the phosphate buffer were collected at a rate of 6 ml. per hour. One ml. of each eluate fraction was dried on an aluminum planchet for determination of radioactivity, and the remaining 1 ml. was analyzed for amino acid concentration by the ninhydrin technique.[16]

A typical elution pattern for the separation of the three basic amino acids is presented in FIGURE 1. The radioactivity of the hydroxylysine and lysine fractions parallels closely the concentration of these amino acids, and a satisfactory resolution of the amino acids was obtained by the described procedure.

The planchets were counted in a gas-flow counter with a thin-window Geiger-Müller tube. Samples were corrected for background and for the counting efficiency of the instrument as determined with a standard C^{14}-reference source. Because the samples were thin, correction for self-absorption was not made. Samples were counted at a level that gave 3.2 to 7.1 per cent standard error.

Calculations

The nitrogen of the TCA extract was converted to protein by the factor of 5.56, since collagen contains 18 per cent nitrogen.[17] The collagen of the ex-

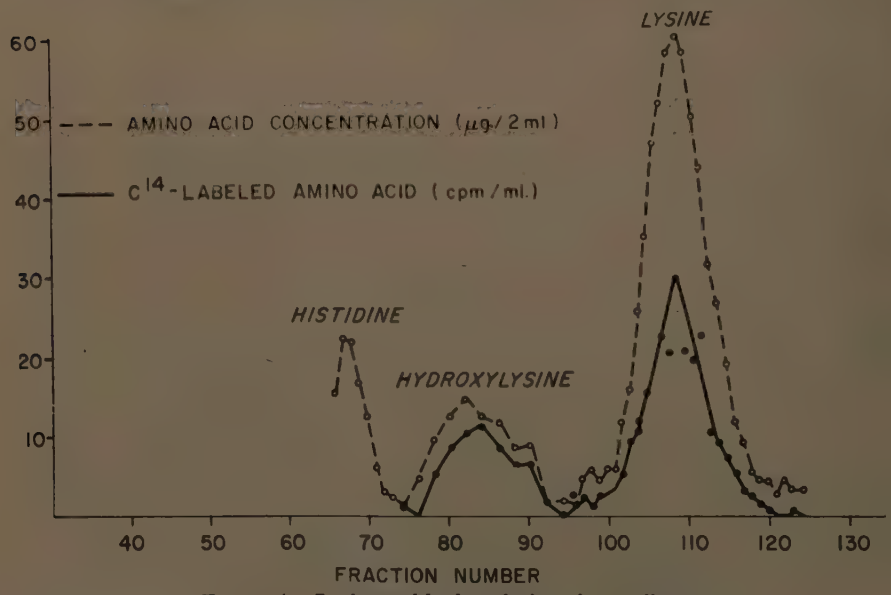

FIGURE 1. Lysine and hydroxylysine of rat collagen.

tract was calculated from the hydroxyproline value by the factor of 7.46 of Neuman and Logan.[14] Values were expressed on a content basis, that is, per gram of dry sponge implant, to eliminate variations due to water content.

In order to calculate the amount of labeled collagen formed from C^{14}-lysine, the specific activity of the hydroxylysine that would result from the hydroxylation of the C^{14}-lysine was calculated from the known specific activity of the labeled lysine and from the molecular weights of the two amino acids. The total counts per minute (cpm) of the hydroxylysine per gram of sponge implant were converted to micrograms of labeled hydroxylysine from its calculated specific activity. The amount of radioactive collagen formed per gram of sponge implant was calculated from the micrograms of labeled hydroxylysine. The factor used for the conversion of hydroxylysine to collagen was based on our experimental mean value for the percentage of hydroxylysine in collagen (1.24), which did not differ significantly from that of 1.3 reported in the literature.[18]

Experimental Groups

Group 1. The *in vitro* hydroxylation of C^{14}-lysine was studied in slices of 34-day-old sponge tissue from 2 male rats and of 24-day-old tissue from a female rat. Approximately 500 mg. of tissue slices was placed in a flask containing 5 ml. of Krebs-Henseleit solution[19] and 1 ml. of a neutralized solution of lysine-2-C^{14} in physiological saline (0.255 mg. DL-lysine-2-C^{14} monohydrochloride or 1 μc.). The flasks were gassed with 95 per cent O_2:5 per cent CO_2, and capped. The aeration was repeated at 2-hour intervals during the 12-hour incubation period (37.5° C.). At the end of the incubation the slices were removed and blotted to remove excess C^{14}-lysine. The tissue slices were then homogenized in physiological saline containing 0.25 per cent lysine. The saline-insoluble residue was removed by centrifugation at 15,000 g for 1 hour and collagen was extracted.

For the study of proline hydroxylation in a cell-free system, sponge connective tissue of 12 days' growth was homogenized in buffer* in the VirTis homogenizer (0.5 min. at minimum speed and 0.5 min. at maximum speed). The cellular particles in the filtrate were fractionated by differential centrifugation by the scheme shown in FIGURE 2. All steps of the fractionation were carried out in the cold. Collagen fibers obtained from the sponge tissue were washed 4 to 6 times with chilled physiological saline (*p*H 7.4) to remove all loosely-bonded noncollagenous protein. Equal aliquots of the washed fiber were placed in Erlenmeyer flasks containing 10 μM of adenosinetriphosphate (Na_2H_2 ATP·$4H_2O$), 20 μM of phosphocreatine (calcium hydrate), 0.95 μM of L-proline-C^{14} (1.31 μc./μM), and 5 ml. of 105,000 g supernatant or filtrate. The cellular particles were suspended in buffer and added according to the scheme indicated in TABLE 1. Each flask contained a final volume of 7 ml. and equal amounts of protein nitrogen and collagen.

The capped flasks were aerated with 95 per cent O_2:5 per cent CO_2 every 2 hours and incubated at 37.5° C. for 5 hours. Following the incubation, the

* Composed of 0.35 M sucrose, 0.035 M $KHCO_3$, 0.025 M KCl, 0.004 M $MgCl_2 \cdot 6\ H_2O$ in *pH* 7.8 phosphate buffer.[20]

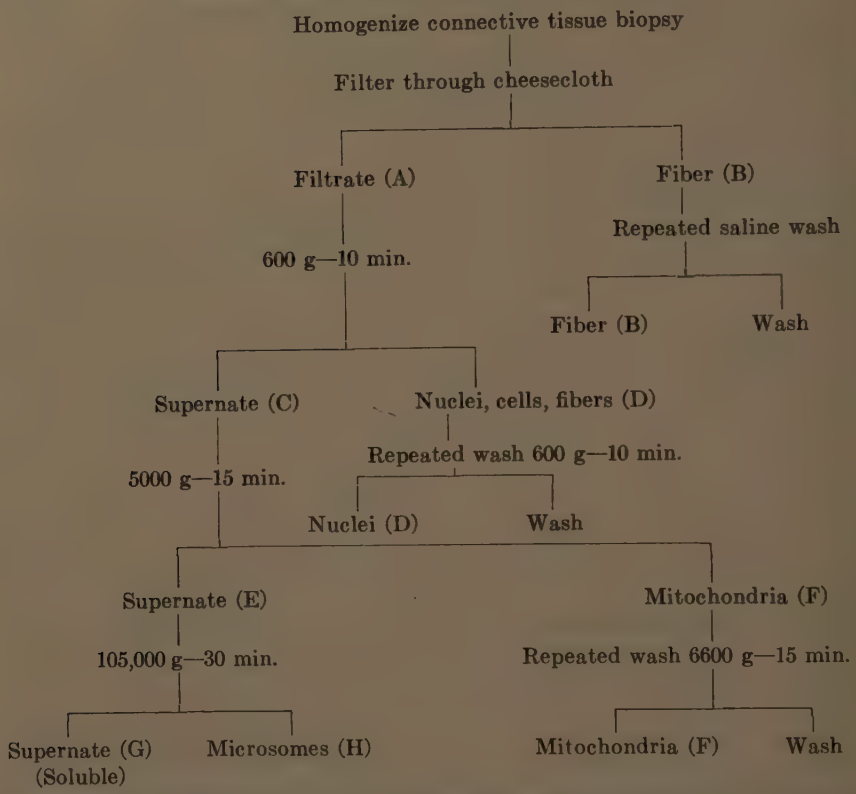

FIGURE 2. Schematic diagram of the differential centrifugation used in the preparation of fibroblasts.

contents of the flasks were dialyzed against distilled water until the water was chloride free. The contents of the dialysis bag were dried and then extracted with hot trichloroacetic acid (90° C.) to solubilize the collagen. The extract was dried, hydrolyzed with 6 *N* HCl, and proline and hydroxyproline were separated by column chromatography according to the method of Robertson.[21] The method involved the deamination of the alpha-amino acids with $NaNO_2$ and ammonium sulfamate in acid. The mixture was desalted on Dowex-50 × 8, and the proline and hydroxyproline were eluted with ammonia. The eluate fractions containing the two amino acids were combined, dried, and placed on a Dowex-50 × 8 column, which then was eluted with HCl. Hydroxyproline was removed with 1.5 *N* HCl, and proline with 4 *N* HCl. Fractions of 2 ml. were collected at a rate of 1 ml./min. The fractions were dried several times to remove HCl and then 2 ml. of H_2O was added. One ml. of each fraction was placed on a planchet and dried for radioactivity determination. The remaining 1 ml. was analyzed either for hydroxyproline[14] or for proline (Robertson, personal communication). The elution pattern of the separation of hydroxyproline and proline is indicated in FIGURE 3.

TABLE 1

IN VITRO HYDROXYLATION OF C^{14}-LABELED PROLINE BY CELLULAR PARTICLES
Rat Connective Tissue, 12-Day-Old, μg. C^{14}-Labeled Hydroxyproline × 10^{-4}/Flask

Cellular particle added	
Male	
Nuclei, mitochondria, microsomes	3.0
Mitochondria, microsomes	5.5
Microsomes	1.5
Nuclei	2.0
Mitochondria	2.2
None	4.2
Female	
None	2.2
None (boiled supernate)	0.9

Flask contained collagen fiber, 105,000 g supernate, ATP + phosphocreatine, C^{14}-L-proline plus added particle.

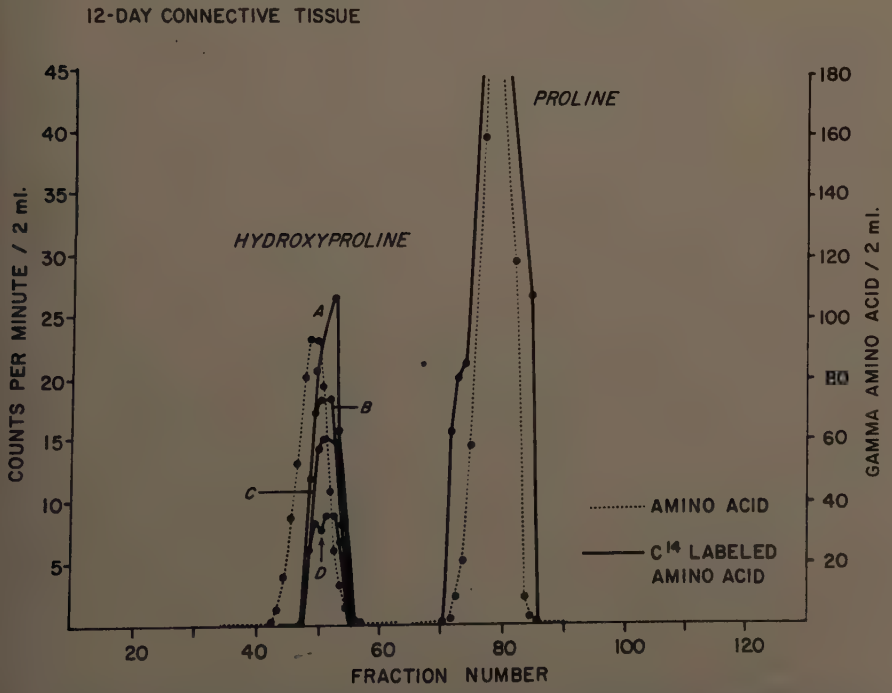

FIGURE 3. Acellular hydroxylation of C^{14}-proline: A, mitochondria plus microsomes; B, 105,000 g only; C, filtrate (nuclei plus mitochondria plus microsomes); D, nuclei, microsomes, and mitochondria singly.

Group 2. In the study on the rate of collagen development in the rat, the sponge tissues were removed at time intervals of from 3 to 300 days after implantation. Tissues from 124 males and 139 females were analyzed for collagen.

Group 3. The rate of the incorporation of C^{14}-lysine into collagen and of its subsequent hydroxylation was followed in 20- and 21-day-old sponge connective tissue from 4 male and 4 female rats, respectively. The animals were given an intraperitoneal injection of 0.012 μc. of DL-2-C^{14}-lysine monohydrochloride (4.7 mg.) per 100 gm. of body weight early on the morning of the twentieth or twenty-first day of tissue growth. The tissues were removed at 0.5, 1, 2, 3, 4, 5, 6, 8, 11.5, 25.5, and 27.5 hours after the injection of the labeled lysine.

The collagen of the tissues was extracted and analyzed for protein, hydroxyproline, lysine, and hydroxylysine. The radioactivity in the lysine and hydroxylysine of the collagen was determined.

Group 4. The effect of tissue age upon the incorporation of C^{14}-lysine and its hydroxylation in collagen was studied in sponge connective tissue removed from female rats after 13, 20, 75, 175, 191, and 261 days of growth, and from male rats after 13, 20, 38, 75, and 175 days of growth. The animals were given a single intraperitoneal injection of 0.012 μc. of DL-2-C^{14}-lysine-HCl (4.7 mg.) per 100 gm. of body weight, and the tissues were removed 16 hours after injection. Tissues of this series were analyzed as described for group 3.

Group 5. The rate of disappearance of C^{14}-labeled collagen was studied in 4 male and 4 female rats in each of which 4 sponges had been implanted 12 days previously. The animals were given a single injection of C^{14}-lysine (0.012 μc./100 gm. body weight), and the first sponge was removed 16 hours later. The remaining sponge tissues were removed from the male 7, 14, and 23 days postinjection and from the female 7 and 23 days postinjection. Tissues were analyzed as described for group 3.

Results and Discussion

Following the sponge implantation, the interstices fill with an eosin-staining proteinaceous material that coalesces into argyrophilic strands. Within 5 days following implantation fibroblasts surround the sponge, forming a collagenous capsule, but a chemically or a tinctorially detectable collagen does not appear within the sponge until the fifth or sixth day. The fibroblasts appear to travel along the coalesced strands that are found lining the pores of the plastic sponge, and by the fourteenth day the sponge is filled by the newly formed fibrocollagenous tissue. As the fibroblasts migrate into the sponge, the fibrinlike strands disappear and are replaced by the interfibrillar substance and the collagen fibers.

In Vitro Hydroxylation of Lysine and Proline

Because of the importance of hydroxylysine and hydroxyproline for collagen structuration, *in vitro* studies were performed to elucidate some of the mechanisms involved in hydroxylation of lysine and proline. Slices of the sponge

TABLE 2

IN VITRO HYDROXYLATION OF LYSINE-2-C^{14} BY BIOPSY CONNECTIVE TISSUE
(Twelve-Hour Incubation)

Sex	Age of tissue	Collagen	
		Hydroxylysine cpm/mg.	μg. C^{14}-collagen/gm. sponge
♂	34	1679	32.4
♂	34	1148	29.3
♀	24	3320	42.9

tissue converted labeled lysine to tagged hydroxylysine (TABLE 2). The incubation of cellular constituents with C^{14}-labeled proline as the parent amino acid indicated that the intact cell was not necessary for hydroxylation and that the supernatant fraction (105,000 g) plus mitochondria and microsomes produced the greatest amount of hydroxylation (FIGURE 3). Addition of mitochondria, microsomes, or nuclei singly to the 105,000 g supernatant not only failed to enhance hydroxylation but appeared to depress it (TABLE 1). The heat lability of the 105,000 g supernatant would suggest that the process of hydroxylation is enzymatic in nature. The combination of all the cellular particulates in the saline filtrate of the 12-day-old sponge tissue resulted in the formation not only of labeled hydroxyproline but of other distinct labeled fractions that may represent metabolic relatives of proline. It is possible that this hydroxylation of proline *in vitro* may represent a *de novo* synthesis of collagen, although this was by no means established.

In Vivo Collagen Formation

The study of the accumulation of collagen in the sponge implants of 124 male and 139 female adult rats indicated a striking sex difference. Collagen was chemically identified within 5 to 6 days following implantation, and rapidly increased during the initial 20 days in both sexes (FIGURES 4 and 5). After the twentieth day the rate of collagen accumulation in the sponge of the female slowly decreased until day 40 (FIGURE 4). At this time the maximum collagen content was attained. From 40 to 300 days, the collagen content slowly decreased at a constant rate. The data during this latter period could be fitted by a linear regression line with a negative slope ($p < 0.01$). In the male sponge tissue (FIGURE 5), the rate of accumulation of collagen beyond day 20 decreased more rapidly than the rate in the female, but the collagen content continued to increase to 300 days.

This interesting sex difference in collagen accumulation prompted a study of the effects of sex and tissue age upon collagen synthesis. The incorporation of lysine-2-C^{14} and its subsequent hydroxylation to labeled hydroxylysine in the collagen of connective tissue were used as an index of collagen synthesis.

The time at which the maximum quantity of labeled hydroxylysine appeared in collagen following a single injection of lysine was determined in 21-day-old

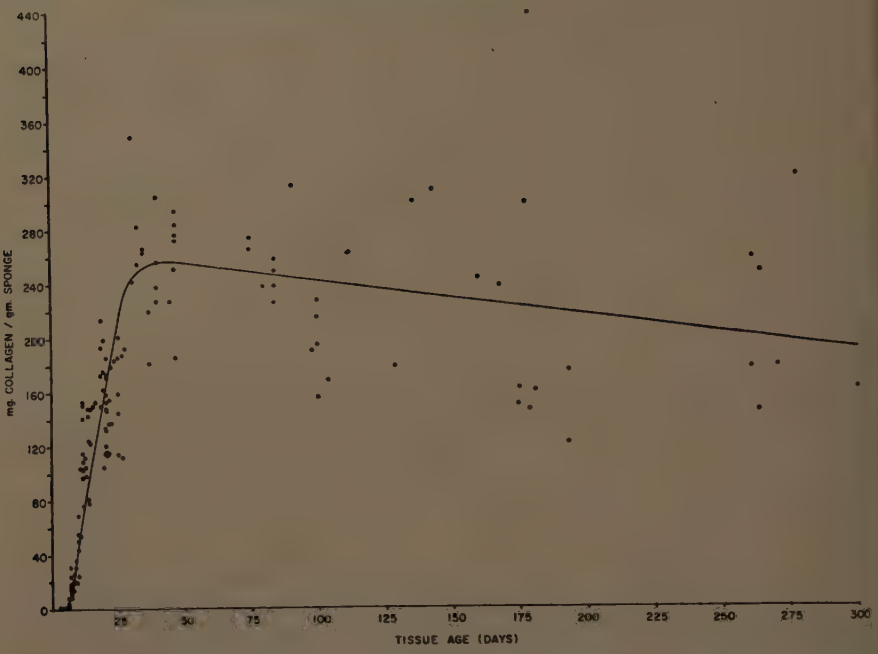

FIGURE 4. The relationship of collagen content to tissue age in sponge connective tissue of the female rat.

tissue of the female rat. Labeled hydroxylysine appeared within 1 hour and reached a maximum (equivalent to 14 μg. of collagen) by 6 to 8 hours (FIGURE 6). There was no apparent decrease in the amount of labeled collagen 27.5 hours after the injection. A similar study of 20-day-old male tissue showed parallel results (FIGURE 7). Maximum labeling of hydroxylysine (equivalent to 17 μg. of collagen) occurred by 6 hours and did not appear to change for as long as 25 hours. In subsequent experiments, sponge tissues were removed 16 hours after the lysine injection, that is, during the period when a constant amount of labeled hydroxylysine was maintained.

Since the chemical measurements of collagen accumulation (FIGURES 4 and 5) indicated a high rate of net synthesis of collagen at 20 days, it was likely that the appearance of labeled hydroxylysine in the collagen was largely the result of *de novo* synthesis as opposed to a metabolic turnover involving partial degradation or exchange of amino acid residues.

Six- to 20-day period. The accumulation curves (FIGURE 4 and 5) indicated a close similarity between male and female in the first 20 days of tissue growth. However, isotopic studies made during this period showed marked sex differences in incorporation patterns of lysine which entered collagen.

The amount of labeled hydroxylysine appearing in the collagen of 12-day-old sponge connective tissue of the male rat 16 hours after the injection of C^{14}-lysine was double the amount appearing in the collagen in female tissue of

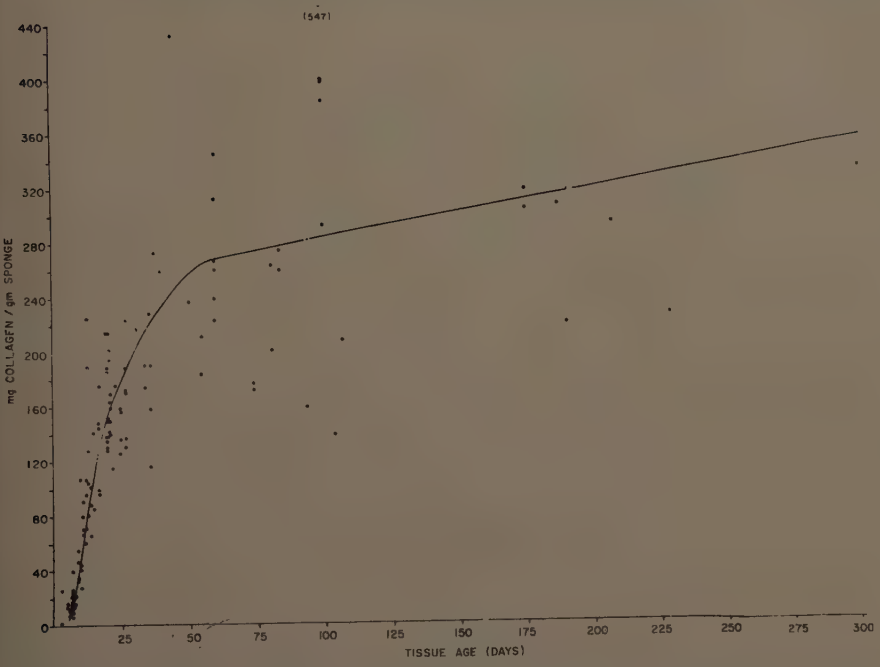

FIGURE 5. The relationship of collagen content to tissue age in sponge connective tissue of the male rat.

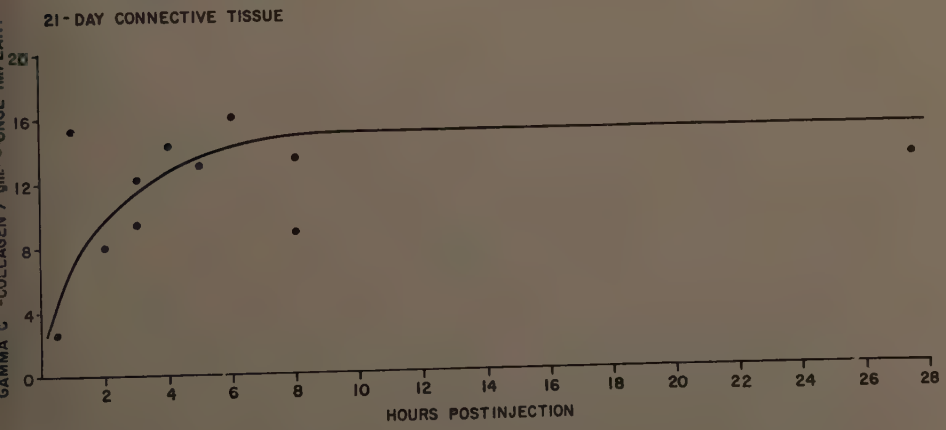

FIGURE 6. The appearance of C^{14}-labeled collagen in the adult female rat.

the same age (TABLE 3). Moreover, a closely parallel occurrence was noted in the *in vitro* studies. The supernatant fraction of 12-day-old male sponge connective tissue hydroxylated twice the amount of labeled proline as did the female supernatant (TABLE 2). Since male and female tissues at day 12 were

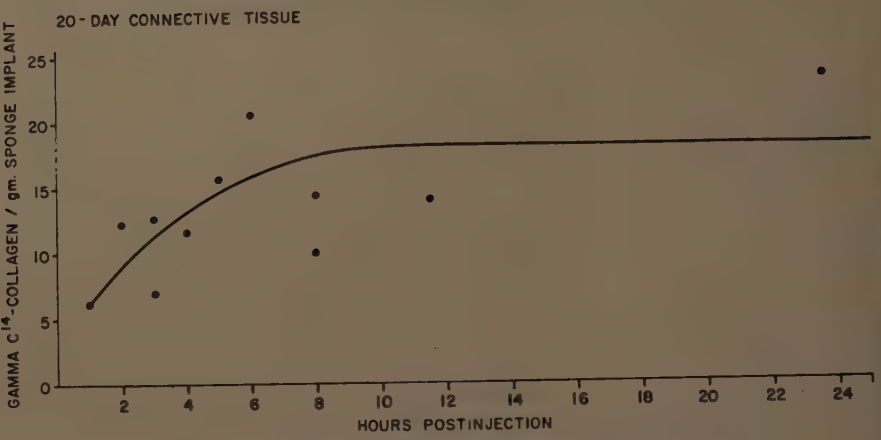

FIGURE 7. The appearance of C^{14}-labeled collagen in the adult male rat.

TABLE 3
DISAPPEARANCE OF C^{14}-LABELED COLLAGEN
Rat Connective Tissue,* μg./gm. Sponge

Body weight (gm.)	Days postinjection			
	0.67	7	14	23
		Male		
315	17	17	13	—
380	—	11	11	10
355	40	17	28	27
385	34	—	—	13
Mean	31	15	17	17
		Female		
204	17	14	—	5
227	12	10	—	4
205	17	14	—	4
200	15	9	—	7
Mean	15	12	—	5

* Initial tissue age, 12 days.

effecting a net synthesis of almost equal amounts of collagen (FIGURES 4 and 5), the male tissue must either have had a large amount of collagen degradation occurring concomitantly with its greater synthesis or the appearance of labeled hydroxylysine in the male collagen reflected not only *de novo* synthesis, but also nonsynthetic turnover of pre-existing collagen.

To distinguish between these two possibilities, the rate of disappearance of labeled hydroxylysine from male and female 12-day-old tissue was followed by removing sponges at 7, 14, and 23 days after lysine injection. It can be

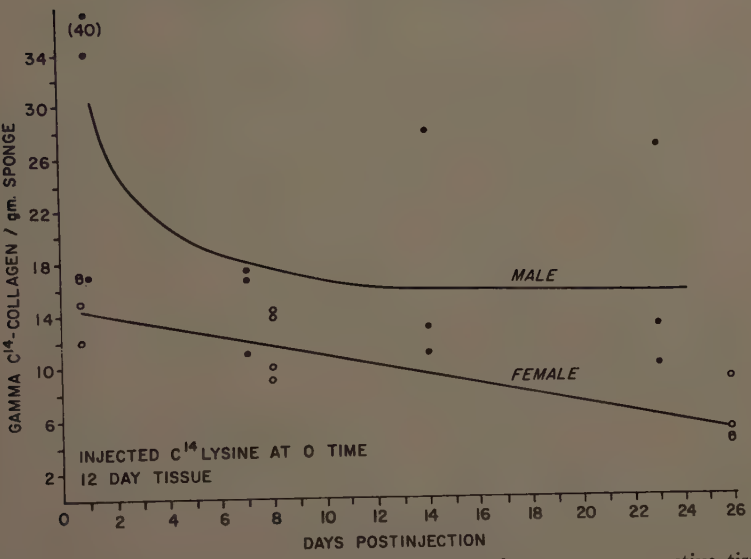

FIGURE 8. The disappearance of C^{14}-labeled collagen from young connective tissue of the rat.

seen from FIGURE 8 that the male collagen rapidly lost labeled hydroxylysine in the first 7 days, while the female collagen lost hydroxylysine at a slower rate in this period. This supports the concept that the 12-day-old male tissue had both a greater amount of synthesis and a greater degradation of collagen than the female tissue.

Following the loss in the first 7 days after lysine injection, the male tissue then lost labeled collagen very slowly, while the female tissue continued to lose labeled collagen at a constant rate over the 23-day period (FIGURE 8). These results are, of course, complicated by the fact that further collagen accumulation occurred during the period of observation. However, it is nonetheless clear that over the 23-day period the female lost a much larger proportion of the collagen formed on day 12 than did the male. The possibility exists that the male collagen may be in 2 compartments, one undergoing rapid degradation, the other being only slowly degraded.

Twenty- to 300-day period. While collagen accumulation appeared to proceed at comparable rates in male and female tissue during the first 20 days of growth (FIGURES 4 and 5), the curves became widely divergent beyond 21 days; the male rate of accumulation fell off more rapidly than did the female rate. To determine possible changes in the rate of synthesis of collagen beyond 20 days, incorporation of labeled lysine and its subsequent hydroxylation were determined in tissues of various ages.

A most interesting result was obtained in that there was an almost constant amount of labeled hydroxylysine found at all tissue ages within each sex (TABLE 4). The mean value for male tissue at ages 20, 38, 75, and 175 days was 16 μg. labeled collagen/gm. sponge, and for the female tissue at 13, 20, 75,

TABLE 4

C^{14}-LABELED COLLAGEN AT DIFFERENT TISSUE AGES: RAT CONNECTIVE TISSUE SIXTEEN HOURS POSTINJECTION, μg./gm. SPONGE

Tissue age (days)	Male	Female
13	31 (3)*	14 (4)
20	15 (8)	13 (8)
38	20 (1)	—
75	13 (2)	15 (2)
175	15 (2)	17 (1)
191	—	18 (1)
261	—	9 (2)

* Number of animals.

175, 191, and 261 days, 15 μg./gm. sponge. Since there was no difference in the incorporation of labeled amino acid into collagen related to the sex or age of the tissue, the divergence of the male and female accumulation curves must be explained on some other basis.

The data in FIGURE 5 indicated a greater rate of collagen accumulation in the male tissue at day 20 than at day 175, yet the incorporation of labeled amino acid was not different at the two ages. If the incorporation of isotope represented only *de novo* synthesis of collagen, then a collagen-degrading mechanism must have developed so that the interaction of synthesis and degradation resulted in a continually decreasing net synthesis of collagen. If the incorporation of isotope represented *de novo* synthesis plus turnover due to partial degradation or exchange, there must have been a gradual shift in the relative contributions of the two types of turnover, so that by day 175 nonsynthetic turnover predominated.

The female curve (FIGURE 4) from day 20 to its maximum at day 40 might be explained in the same manner as the male curve. However, there was a marked difference in the shape of these two curves; the female curve rose at a more rapid rate and leveled off quite early. Either the degradation mechanism came into play later in time, but greater in magnitude than in the male tissue, or else *de novo* synthesis gave way to other types of turnover much earlier than in the male.

Beyond day 40 the collagen of the female tissue was gradually reduced, in contrast to the male collagen, which continually increased. If the cessation of collagen accumulation at day 40 in the tissue of the female represented an equilibrium between *de novo* synthesis and degradation, then beyond day 40 degradation must have exceeded synthesis. If the cessation at day 40 represented complete absence of *de novo* synthesis, then beyond day 40 a small constant degradation must have come into play. At day 261, the incorporation of labeled amino acid into collagen had dropped from the level prevailing from day 13 to 191, suggesting a reduction in the activity of the fibroblasts in the older tissue. Such a reduction would be in keeping with the change in the morphology of the fibroblast with tissue age; that is, the cell becoming narrower and exhibiting a nuclear thinning and a decreased amount of cytoplasm.

The available data do not permit the assignment of an unequivocal biochemical mechanism to explain the shape of the collagen accumulation curves, but there can be no doubt that there is a considerable metabolic turnover of collagen in the sponge connective tissues even of older ages. It is interesting to note that the sponge tissue contains a number of active proteases and peptidases, including prolidase and prolinase. These enzymes reached their maximum levels after the rate of collagen accumulation had passed its peak and just at the time when degradation or turnover mechanisms must have become maximal.

The magnitude of collagen turnover indicated by these studies does not agree with the concept of relative metabolic inertness of collagen demonstrated by Neuberger's studies on the rat skin and tendon collagen.[22] The sponge tissue collagen appears to be much more active metabolically; in fact, it bears a close resemblance to the collagen found in the involuting rat uterus[23] and in the tissue formed in response to Irish moss injection,[24] both of which have rapid turnover rates.

The changes in arteries associated with aging consist of the formation of collagen in the intima and a localized and more marked development of fibrocollagenous tissue at the sites of hemodynamic trauma. There is also a striking sex difference in the development of these vascular changes. The findings presented on the biochemical studies of connective tissue from male and female rats may form a basis for further elucidation of the aging processes and related sex differences.

Summary

In sponge-induced growth of fibrocollagenous tissue in rats, the male and female produced tissues that differed in their metabolism of collagen. This sex difference, probably related to variations in the metabolic processes of the fibroblast, resulted in the eventual formation of a greater concentration of collagen in the male sponge tissue than in the female. Furthermore, the male tissue continued to accumulate collagen out to a tissue age of 300 days, while in the female tissue the collagen concentration gradually diminished after passing through a maximum at 40 days.

A sex difference in fibroblastic activity was apparent in the 12-day-old tissue. Following the single injection of C^{14}-lysine, the collagen of the male tissue had twice the amount of labeled hydroxylysine as that of the female. The hydroxylation of C^{14}-proline in the cell-free system from the male tissue was also twice as great as in that of the female.

There appeared to be a fairly constant turnover of collagen of similar magnitude in both male and female tissues beyond 20 days of tissue age. This turnover may be related to the proteases and peptidases present in the sponge tissue.

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HORMONAL CONTROL OF PERMEABILITY AND MOBILIZATION OF FAT DEPOTS

Joseph Seifter, David H. Baeder
Wyeth Institute for Medical Research, Radnor, Pa.,

Chris J. D. Zarafonetis, John Kalas
Department of Medicine, Temple University School of Medicine, Philadelphia, Pa.

Various hormones can alter the permeability of the ground substance. Most methods for demonstrating such an effect are based on the spreading reaction of Duran-Reynals and represent deviation from the normal spread of injected dyes or colloidal material. The connective tissue sites selected for such studies contain hyaluronic acid or similar mucopolysaccharides. These are attacked by hyaluronidase and offer a convenient site for demonstrating either increased spreading or antihyaluronidase activity. Quantitative methods require technically elaborate procedures, such as measurement of passage of dye across the synovial membrane.[1]

The effects usually demonstrated are acute and do not necessarily indicate what may occur on chronic administration of steroids and other hormones. Thus Baker and Whitaker[2] and Hayes,[3] among others, have demonstrated that the antipermeability effects seen with single injections of cortisone cannot be demonstrated with repeated injections. Chronic administration may result in enhanced spreading. It has been shown that short-term administration of hydrocortisone results in the production of a more viscous synovial fluid.[4, 5] The increased viscosity presumably reflects a higher degree of polymerization of the mucopolysaccharides present in the connective tissue and would decrease permeability. It has also been demonstrated that administration of cortisone suppresses the synthesis of mucopolysaccharides, particularly chondroitin sulfate.[6] The suppression of synthesis can therefore effect a depletion of mucopolysaccharides in connective tissues and result in enhanced permeability.

The mechanism by which various steroids and hyaluronidase cause permeability changes has not been elucidated. The original concepts involved weakening of the barrier to account for increased permeability or enhancing the barrier to bring about decreased permeability. The weakening or strengthening of the barrier was a reflection of changes in the viscosity of the mucopolysaccharides present in the ground substance. Recently, more dynamic functions have been assigned to the mucopolysaccharides, so that they are considered either as protective colloids or as ion exchangers; in this fashion they would actively participate in dispersion or transport phenomena. Our investigations of the role of the degree of polymerization of hyaluronate are depicted in the tables that illustrate this paper.

Table 1 presents data showing the effect of hyaluronidase and 3 different hyaluronates on the spreading reaction. Increasing within limits the amount of hyaluronidase injected with the dye resulted in increased spreading; none of the hyaluronates at lower concentrations had significant effect and, at higher concentrations, they were significantly inhibiting. The antipermeability effect

TABLE 1

EFFECT OF HYALURONIDASE AND VARIOUS POLYMERIZED HYALURONATES ON SQ. MM. SPREAD

(End of 1 Hour)

Mg./ml.	0.005	0.01	0.1	0.2	0.5	1.0	2.0
Hyaluronidase	—	197.2	388.2	483.0	643.6	675.6	587.2
Streptococcal hyaluronate	171.7	217.2	198.1	163.7	100.3	—	—
Umbilical cord hyaluronate	160.7	193.5	202.5	149.5	112.9	—	—
Vitreous humor hyaluronate	202.2	194.7	171.5	142.3	138.3	—	—

TABLE 2

EFFECT OF PARTIALLY DEPOLYMERIZED HYALURONIC ACID (PDHA) ON SPREADING

	Area of spread (sq. mm.) at 60 min.
Saline	160
1 Per cent hyaluronidase	375
2 Per cent hyaluronidase	480
5 Per cent PDHA	550

may have been due simply to the greater viscosity of the solution injected. The results obtained with hyaluronidase suggested the possibility that depolymerized hyaluronate might be the active means by which the enzyme brought about enhanced spreading. We therefore prepared depolymerized hyaluronate by incubating streptococcal hyaluronate with testicular hyaluronidase, autoclaved the solution at 104° C., and centrifuged it. The supernatant was injected into rabbits. The results are shown in TABLE 2. It will be seen that the depolymerized hyaluronate was as effective a spreading agent as was the enzyme.

The effect of hyaluronidase on the permeability of blood vessels and tissues to circulating lipids of hyperlipemic animals is shown in TABLE 3. During the first 5 weeks of treatment the hyaluronidase-treated rabbits had consistently lower blood cholesterols than those administered cholesterol without hyaluronidase. After the fifth week there was an escape from the hypocholesterolemic action. The reasons for this are not clear but, coincidentally, there was a marked elevation of antihyaluronidase titer in the blood of the hyaluronidase-treated rabbits. The atheromata in the aorta were at least as severe in the latter group as in those receiving only cholesterol; in some instances they were considerably more severe. There was also a marked deposition of fat in the liver, kidneys, and spleen of the hyaluronidase-treated rabbits. These findings indicate that hyaluronidase increased the permeability of various tissues for lipid. A similar effect has also been reported by Cali.[7] Enhancing effects of hyaluronidase have also been reported by Wong *et al.*[8] in rabbits rendered excessively hyperlipemic by the combined administration of cortisone and cholesterol. Hyaluronidase lowered the blood lipids, but enhanced atherom-

TABLE 3

EFFECT OF HYALURONIDASE ON TOTAL BLOOD CHOLESTEROL LEVELS AND AORTIC LESIONS OF RABBITS ON AN ATHEROGENIC REGIMEN

Treatment	Total blood cholesterol (mg. %)								Degree of atheromatosis of aorta	
	Weeks of experiment									
	0	1	2	3	4	5	6	7	Thoracic	Abdominal
Control	82	81	70	95	93	89	98	75	0	0
5 Per cent cottonseed oil	70	81	87	98	84	85	103	88	0	0
5 Per cent cottonseed oil + 0.5 per cent cholesterol	96	303	155	178	209	244	496	377	1.6	1.8
5 Per cent cottonseed oil + 1000 TRU hyaluronidase/kg. S.C./day	72	86	96	93	76	76	97	104	0.5	0.2
5 Per cent cottonseed oil + 0.5 per cent cholesterol + 1000 TRU hyaluronidase/kg. S.C./day	72	144	96	114	121	141	267	641	2.1	2.6

TABLE 4

INHIBITOR OF LIPEMIA CLEARING

Effect of CF and CF + CFI Rat Plasma on Lipemic Dog Plasma (LP)

No. rats CF*	Source of CF	No. rats CFI†	Source of CFI	Light transmission (average percentages)		
				LP	LP+CF	LP+CF +CFI
6	Heparin	15	Cortisone	9	96	10
6	PDHA	6	Cortisone	22	86	22
6	DCA	6	Cortisone	20	97	21
6	Heparin	6	Stress	20	96	21
6	Heparin	4	0.1 AKS‡	18	86	41
6	Heparin	3	0.2 AKS‡	23	86	27
6	Heparin	5	0.4–0.8 AKS‡	21	86	23

* CF = lipemia clearing factor.
† CFI = lipemia clearing factor inhibitor.
‡ Ml. antikidney serum/100 gm. of rat.

atous lesions and resulted in marked deposition of lipids in the liver. Adlersberg *et al.*[9] and Dury and DiLuzio[10] have reported that, although cortisone elevated cholesterol-induced hyperlipemia in rabbits, it did not enhance the formation of atheromas; this might be due to the antipermeability effect of cortisone.

The effect of hyaluronidase on plasma lipids suggested that a lipid-clearing factor was involved. TABLE 4 presents data demonstrating that plasma from

TABLE 5
EFFECT OF DCA ON LM HYPERLIPEMIA IN RATS

Treatment	Intact		Adrenalectomized		Hypophysectomized	
	CH	FA	CH	FA	CH	FA
None	78	100	79	92	72	90
LM	199	304	189	297	190	340
LM + I*	78	92	71	85	73	92
LM + A†	81	99	79	93	71	99
LM + H‡	80	90	85	91	70	100

* I = plasma from intact rats treated with DCA.
† A = plasma from adrenalectomized rats treated with DCA.
‡ H = plasma from hypophysectomized rats treated with DCA.

rats administered partially depolymerized hyaluronate or desoxycorticosterone caused delactescence when added to lipemic plasma. The clearing was not associated with lipolysis and therefore was not due to lipoprotein lipase.[11] A more direct effect of desoxycorticosterone acetate (DCA) on blood lipids *in vivo* is shown in TABLE 5. The hyperlipemia was induced by injection of a lipid-mobilizing substance (LM) to be described below. DCA was injected into intact, adrenalectomized, and hypophysectomized rats. Two hours later the animals were bled and the plasmas separated. The plasma from the intact rats was coded I, that from adrenalectomized ones as A, and that from hypophysectomized ones as H. Another group of rats received injections of LM. One hour later 25 per cent of the rats were administered either I, A, or H intravenously. The same experimental design was employed for adrenalectomized and hypophysectomized rats. One hour after the injection of DCA plasma the rats were anesthetized and plasma obtained for lipid analysis. It will be seen that DCA plasma had no effect on the blood lipids of untreated rats, but that DCA plasma abolished the hyperlipemic action of LM. The DCA plasma was effective whether obtained from intact, adrenalectomized, or hypophysectomized rats. It may be assumed that the antagonistic effect was peripheral and probably at the site where LM acts. The possibility that this effect is caused either by retention of the mobilized lipids by the liver or by demobilization by altering permeability of the fat depots is being investigated. An effect of DCA on blood lipids in dogs has been reported by DiLuzio *et al.*[12]

Since the effect of DCA in hyperlipemic rats resembled that of hyaluronidase in hyperlipemic rabbits, it was of interest to investigate the effect of partially depolymerized hyaluronic acid (PDHA) on the hyperlipemia induced by LM or by plasma from patients with familial hyperlipemia (AA and DW). The antihypercholesterolemic action of PDHA in nephrotic rats has already been reported.[13] The data are presented in TABLE 6. The injection of LM or plasma from hyperlipemic patients produced hyperlipemia in rats in 1 hour, and the injection of 5 mg. of PDHA/kg. intravenously 1 hour later restored the blood lipids to normal values. The effect of PDHA was similar to that of DCA and hyaluronidase. PDHA is a nonsulfated mucopolysaccharide. Similar non-

TABLE 6
EFFECT OF PDHA ON HYPERLIPEMIA IN RATS

Treatment	FA	CH
LM	326	272
AA	299	246
DW	306	285
LM + normal human plasma	338	280
LM + PDHA	94	82
AA + PDHA	103	96
DW + PDHA	92	85

sulfated partially depolymerized mucopolysaccharides are probably activated by administration of hyaluronidase or DCA. It has already been mentioned that these agents release a delactescing factor that differs from lipoprotein lipase in not exhibiting lipolytic activity. We have reported similar delactescing properties for other nonsulfated mucopolysaccharides.[11, 13a] The antihyperlipemic and delactescing properties of nonsulfated mucopolysaccharides differ from those of sulfated mucopolysaccharides such as heparin. The latter release lipoprotein lipase, which is not always associated with delactescence or hypolipemic action. The most striking instance of this is the failure of heparin to affect the lactescence or degree of lipemia in rabbits subject to repeated bleeding[14] or in fats injected with LM.

In 1954 we reported that the delactescing action of heparin-clearing factor was inhibited *in vitro* by the addition of plasma from animals administered cortisone, exposed to cold, or subjected to the nephrotic syndrome;[12] the data are presented in TABLE 4. The clearing factor was from rats administered 4 mg. heparin/kg. intravenously, and the substrate was plasma from alimentary hyperlipemic dogs. The column headed LP shows the opacity of the lipemic plasma. LP + CF shows that the CF was actively delactescent. The last column shows that plasma from cortisonized rats, rats exposed to cold, or those receiving antikidney serum, when added to an active clearing system, prevented the usual increase in light transmission. In other experiments we established the presence of an *in vitro* clearing factor inhibitor in plasma, centrifuged at 12 g, obtained from human nephrotics, familial hyperlipemics, surgical patients, and obstetrical patients.[15] Several other laboratories have since reported a similar inhibitor under the following conditions: familial hyperlipemia in humans,[16] administration of cortisone,[17, 17a] administration of toxic doses of alloxan,[18] and frequent bleeding of rabbits.[14] The occurrence of a dialyzable inhibitor has now also been reported as present in normal bovine plasma.[19]

Injection of clearing factor inhibitor (CFI) into a variety of species, including humans, resulted in hyperlipemia. As will be shown later, the hyperlipemia did not depend on clearing factor inhibition *in vivo*, but was due to lipid mobilization from the depots. We have therefore dropped the term CFI in favor of lipid mobilizer (LM). The data are shown in TABLE 2. Chalmers *et al.*[20] have isolated from the urine of humans a similar peptide with lipid-mobilizing properties.

TABLE 7
HYPERLIPEMIA INDUCED BY 50 μ PIROMEN/KG. I.V. IN RATS
(Two Hours Postinjection)

	Plasma lipid levels (mg. %)		
	Cholesterol	Fatty acids	Lipid P
Intact	180	210	10
Adrenalectomized	72	108	6
Hypophysectomized	70	115	6

TABLE 8
HYPERLIPEMIA INDUCED BY 1 MG. DFP/KG. I.V. IN RATS
(Two Hours Postinjection)

	Plasma lipid levels (mg. %)		
	Cholesterol	Fatty acids	Lipid P
Intact	190	260	11
Adrenalectomized	62	110	5
Hypophysectomized	58	110	7

Since LM was first demonstrated in rats receiving cortisone or exposed to stress, we determined the role of the adrenals in releasing LM. Bacterial pyrogens or convulsions were used as stressors in adrenalectomized or hypophysectomized rats. TABLE 7 shows that a pyrogenic reaction produced hyperlipemia in intact rats, but not in adrenalectomized or hypophysectomized ones, although fever was present in all groups.

TABLE 8 shows that diisopropylfluorophosphate (DFP) produced hyperlipemia in intact rats, but not in those adrenalectomized or hypophysectomized, although convulsions occurred in all groups. Nonconvulsant doses of DFP did not produce hyperlipemia in intact rats. DFP was originally used because Shore and his colleagues showed that it was a potent inhibitor of lipoprotein lipase.[21] The subconvulsant doses we employed were calculated to give plasma concentrations considerably in excess of those found to inhibit lipoprotein lipase *in vitro*. The results of this experiment made it doubtful that LM caused hyperlipemia by *in vivo* neutralization of lipoprotein lipase. They also indicate that *in vitro* effects of inhibitors cannot be transferred to *in vivo* phenomena. The findings suggested that the hyperlipemia was of more complex origin and that it might be another manifestation of the general adaptation syndrome via the anterior pituitary and adrenal cortex. Testing this hypothesis resulted in the surprising observation that injection of cortisone into hypophysectomized rats failed to release LM. It was then necessary to consider the possibility that the posterior pituitary played a role in the release of LM. TABLE 9 shows the lipemia-inducing properties of dialyzate from the posterior pituitary of hogs. No such activity was demonstrable for Pitocin, Pitressin, extracts of

TABLE 9

EFFECT OF 1 MG. DIALYZATE OF HOG POSTERIOR PITUITARY LOBES/KG. I.V. ON PLASMA LIPID COMPONENTS OF VARIOUS SPECIES

Species	Time in hours postinjection																	
	0			1			2			4			6			24		
	CH	FA	LP	CH	FA	LP	CH	FA	LP	CH	FA	LP	CH	FA	LP	CH	FA	LP
Intact rats (10/hr.)	62	104	5	196	212	9	262	303	10	302	353	12	348	392	12	184	212	8
Hypophysectomized rats (20/hr.)	58	92	5				242	303	11									
Adrenalectomized rats (20/hr.)	64	88	5				258	312	11									
Mice (10/hr.)	52	95	5	195	208	8	312	340	12	402	386	15	440	490	10	120	212	8
Guinea pigs (10/hr.)	85	102	5	186	195	9	245	260	10	322	330	11	385	402	13	84	92	6
Rabbits (12/sample)	62	90	5	190	262	9	240	304	11	320	386	13	360	412	14	75	142	8
Dogs (6/sample)	120	160	6	290	340	11	380	400	18	412	400	19	402	440	18	196	220	9

CH = total plasma cholesterol; FA = total fatty acids; LP = lipid phosphorus. All values in mg. %.

TABLE 10
HYPERLIPEMIA BY 10 MG. PROTAMINE SO_4/KG. I.V. IN RATS
(One Hour Postinjection)

	Plasma lipid levels (mg. %)		
	Cholesterol	Fatty acids	Lipid P
Intact	220	275	12
Adrenalectomized	72	130	6
Hypophysectomized	70	130	6

anterior lobe, or skeletal muscle. The inhibitory action of the adrenals and pituitary on the heparin-clearing action has recently been reported by Pinter *et al.*[22] Hypophysectomy has been reported by Heymann and Haekel[23] and by Bally and Neema[24] either to abolish or largely to prevent the hyperlipemia of experimental nephrosis.

The experience with DFP suggested investigation of the mechanism by which 3 other hyperlipemia agents act. Two of these, protamine[25] and toluidine blue,[26] are considered to be specific antagonists of heparin, and they are supposed to produce hyperlipemia by inactivating the heparin moiety of lipoprotein lipase. The third hyperlipemic agent is phenylhydrazine.[27,28] TABLE 10 shows that protamine hyperlipemia depends upon the presence of the adrenals and pituitary. In this respect it resembles other stressor or toxic agents. Were it acting peripherally on tissue lipoprotein lipase, protamine would be expected to produce hyperlipemia in the absence of these endocrines. Shotz and Page[29] have recently noted identical results obtained in a similar experiment with protamine in hypophysectomized and adrenalectomized rats. We have found that the toluidine blue hyperlipemia occurs only after repeated administration of the dye, and that it is not due to antiheparin action, but to anemia and hepatotoxicity. Phenylhydrazine is not a heparin antagonist; it produces lipemia by a mechanism similar to that of toluidine blue. In our experience, substances that act as stressors and that at the same time are hepatotoxic can produce lipemia in the animal to which they are administered. Stressors without hepatotoxic potential release LM whose lipemic action can be demonstrated in animals sensitized by hepatotoxins.

LM differs from the other agents discussed in that it produces hyperlipemia in the absence of either the adrenals or pituitary, as may be seen in TABLE 9. These data suggest that the hyperlipemic action of LM may be due to a direct neutralization of clearing factor either in the circulation or in the tissues. As mentioned above, considerable doubt is cast on this viewpoint by the fact that DFP does not induce hyperlipemia in concentrations that abolish the lipolytic activity of lipoprotein lipase. More direct studies suggest that LM mobilizes triglycerides, and that the liver determines whether this will be manifested as peripheral hyperlipemia.

The role of the liver on the consequences of mobilization of fat by LM in rats has been described in detail.[30] Animals sacrificed at 0 hour were controls

TABLE 11
ROLE OF LIVER ON FAT MOBILIZATION IN DOGS

		Time (hours) postinjection of LM											
		0			¼			1			2		
		Plasma lipids (mg. %)											
		CH	FA	LP	CH	FA	LP	CH	FA	LP	CH	FA	LP
Fasted dogs with normal liver	Superior mesenteric vein (prehepatic)	60	140	4	62	310	4	58	325	5	58	350	5
	Posthepatic vena cava	82	148	6	92	160	6	100	150	5	100	152	5
Fasted dogs with damaged liver	Superior mesenteric vein (prehepatic)	52	130	3	56	325	3	70	400	6	75	400	6
	Posthepatic vena cava	82	150	6	90	162	6	175	300	6	300	325	9

CH = total cholesterol; FA = total fatty acids; LP = lipid phosphorus.

that had not received LM. At this time the liver had lipid values considerably higher than those reported in the literature for the Wistar strain and were considered to be fatty, although they were not so designated by microscopic examination. The plasma values were within the normal range. Fifteen minutes after injection of LM there was a significant increase in the total fatty acids of the liver that was associated with significant changes in neither the cholesterol nor lipid phosphorus, nor with changes in any of the lipid components of the plasma. One hour following injection, the total fatty acid content of the liver had returned to preinjection level and was associated with significant lowering of cholesterol and lipid phosphorus. All lipid values in the plasma were elevated. The liver lipid values remained depressed for the next 3 hours; during this time the hyperlipemia was intense. As the hyperlipemia subsided, the values for the liver lipids increased, particularly those for total fatty acids. The data suggest that LM promptly mobilized neutral fat to the liver. The latter did not filter this, but added cholesterol and phospholipid, and peripheral hyperlipemia resulted. The data do not indicate whether the mobilized fat was utilized.

The role of the liver in lipemia by LM in dogs is shown in TABLE 11. Samples of blood taken from the superior mesenteric vein and posthepatic inferior vena cava of fasted dogs with no signs of impaired liver function showed an elevation of neutral fats in the blood brought to the liver following injection of LM. The portal hypertriglyceridemia was not manifested as peripheral hyperlipemia. This is in contrast to dogs that had been exposed to chlordan. In these animals LM also induced portal hypertriglyceridemia; this was manifested as peripheral hyperlipemia to which the liver had apparently added cholesterol and phospholipid.

It has already been stated that a variety of stresses release LM into the circulation, and evidence for similar hormonal control in humans is presented in TABLES 12 and 13. TABLE 12 shows that surgical procedures resulted in a marked elevation of total fatty acids in the portal circulation; this was not manifested as a significant hyperlipemia in the peripheral circulation. The

TABLE 12

ARTERIOVENOUS DIFFERENCE IN PLASMA LIPIDS IN OMENTAL CIRCULATION*

Case	Preoperative cholesterol—total fatty acids						Postoperative cholesterol—total fatty acids					
	A	V	A/V per cent	A	V	A/V per cent	A	V	A/V per cent	A	V	A/V per cent
1	228	212	93	280	284	100	280	202	73	308	499	160
2	238	202	85	252	268	83	290	284	98	326	852	262
3	252	228	90	274	286	104	251	220	88	290	446	154
4	226	202	90	312	314	100		226			413	
5	232	201	86	338	312	93	248	209	84	360	440	122
6	312	302	96	348	326	94	346	338	97	402	648	162
7	186	154	83	214	218	100	196	184	94	232	386	166
8	186	142	76	214	208	98	196	188	96	394	786	199

* Mg. %.

TABLE 13

EFFECT OF SURGICAL STRESS ON CLEARING OF LIPEMIC DOG PLASMA *IN VITRO* BY HEPARIN-CLEARING FACTOR PLASMA

Change in optical density

	Time in hours				
	0	1/4	1/2	3/4	1
LP + HCF	0.70	0.49	0.31	0.28	0.28
LP + HCF + LM	0.75	0.73	0.72	0.72	0.72
LP + HCF + prehep. (start of operation)	0.69	0.50	0.28	0.26	0.26
LP + HCF + posthep. (start of operation)	0.69	0.51	0.31	0.30	0.30
LP + HCF + prehep. (end of operation)	0.88	0.80	0.79	0.78	0.78
LP + HCF + posthep. (end of operation)	0.78	0.75	0.74	0.74	0.74

TABLE 14

PLASMA LIPID LEVELS* AT DELIVERY

Maternal (CV)		Cord		Baby	
CH	FA	CH	FA	CH	FA
242	260	90	196	90	166
307	348	79	99		
286	342	82	109		
287	311	95	112		
274	296	85	98		
303	358	72	88		
288	302	81	99		
272	303	84	96		
292	334	76	91		
282	344	84	99	78	104
244	380	90	104		
197	312	71	86		
236	374	99	130		
199	426	76	97	82	91
197	288	71	90	73	104
328	628	103	158	102	174
248	292	87	112	71	86
199	212	84	112	82	91
292	318	102	114	100	112
290	341	82	91	71	103
302	358	78	96	80	93
308	399	82	93	88	99
212	296	78	103	81	92

* Mg. %.

portal hypertriglyceridemia was sometimes evidenced visually from the marked lactescence of the plasma. The samples were from the gastroepiploic vein and gastroepiploic artery. The arterial blood is representative of peripheral or posthepatic circulation, and the venous blood is representative of portal or prehepatic circulation. In other experiments the samples of peripheral circulation were obtained from the cubital vein, and the results in these experiments

TABLE 15

PLASMA LIPID LEVELS* DURING CESAREAN SECTION

Maternal								Cord		Baby	
RA		CV		US		FV		CH	FA	CH	FA
CH	FA	CH	FA	CH	FA	CH	FA				
291	346	282	408	280	392			81	92		
288	342	278	314	288	342			78	84		
		394	395	400	457	400	457	109	121	92	103
360	490	356	484	382	501			99	113	92	110

RA = radial artery; CV = anticubital vein; US = uterine sinus; FV = femoral vein.
* Mg. %.

TABLE 16

EFFECT OF MATERNAL, CORD, AND FETAL BLOOD ON LIPEMIA CLEARING *IN VITRO*

Change in optical density

	Time in hours				
	0	¼	½	¾	1
LP + HCF	0.72	0.41	0.28	0.20	0.18
LP + HCF + LM	0.77	0.76	0.75	0.75	0.75
LP + HCF + cord	0.73	0.64	0.63	0.63	0.63
LP + HCF + maternal cubital vein	0.73	0.68	0.65	0.64	0.64
LP + HCF + uterine sinus	0.75	0.69	0.68	0.66	0.66
LP + HCF + maternal femoral artery	0.75	0.74	0.74	0.74	0.72
LP + HCF + baby femoral vein	0.76	0.75	0.74	0.73	0.72

were no different from those shown in TABLE 12, which also reveals a slight arteriovenous difference of cholesterol suggestive of a possible pathway for cholesterol excretion through the intestines. None of the patients had LM preoperatively in any of the samples as measured by *in vitro* inhibition of delactescence. Following the surgical procedure all specimens contained such activity. The data are presented in TABLE 13.

The liver is not the only organ capable of removing a large quantity of lipid. It is well established that hyperlipemia occurs in pregnant females, but not in the newborn. The cholesterol and total fatty acids of maternal venous blood and umbilical cord blood in humans shown in TABLE 14 are in agreement with published data. More direct measurements obtained by simultaneously sampling maternal blood from the cubital vein, uterine sinus, umbilical cord, and newborn femoral vein blood are shown in TABLE 15. Maternal, cord, and newborn blood contained LM, and TABLE 16 shows the *in vitro* clearing inhibition. It is interesting to note that the newborn circulation contained LM. The human newborn does not have mesenteric or omental fat depots; either these have not yet been deposited or they have been depleted by LM.

The data presented thus far appear to establish an endocrine control of lipid mobilization. The adrenal cortex, the anterior pituitary, and the posterior pituitary are involved. The pathway appears to be a common one for immediate mobilization of lipids during a catabolic process. The sequence is anterior pituitary, adrenal cortex, posterior pituitary, and release of LM. The target for LM is the mesenteric fat depot, which responds with increased permeability and liberation of triglyceride into the portal circulation.

Summary

An endocrine control of lipid mobilization is demonstrated. Cortisone, stress, and various clinical states release a lipid-mobilizing hormone (LM) from the posterior pituitary.

LM releases triglycerides from the mesenteric fat depots to the liver.

Peripheral hyperlipemia results when the liver is unable to utilize the triglyceride load. The liver contributes the cholesterol and lipid phosphorus of the hyperlipemia.

LM hyperlipemia is antagonized by partially depolymerized nonsulfated mucopolysaccharides, but not by heparin.

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EFFECTS OF HORMONES ON CONNECTIVE TISSUE AND MUCOPROTEINS*

Norman F. Boas
Research Division, Norwalk Hospital, Norwalk, Conn.

The endocrine glands play a vital role in the homeostasis and metabolism of connective tissue. Including special situations, it may be said that almost all the known hormones are capable of stimulating connective tissue. One need only cite the stimulatory effects of androgens on the cock's comb,[1, 2] estrogens on the sex skin of the monkey,[3] estrogens and relaxin on the symphysis pubis,[4] and thyrotropic hormone on the production of exophthalmos in the *Fundulus*[5] and the guinea pig.[6] Clinically, in pituitary and thyroid deficiencies one sees myxedema of the connective tissue that can be reversed by the administration of thyroxin. Although blood vessels are a specialized form of connective tissue, it is most probable that endocrine factors regulate their metabolism as well. Much evidence has been cited in this regard.

We have been particularly interested in the endocrine factors that regulate blood and tissue levels of hexosamine-containing compounds. Hexosamine (glucosamine and galactosamine) are widely distributed in the body and account for approximately 0.1 per cent of body weight.[7] They do not occur in a free state, but as components of nucleoproteins, mucoproteins, and mucopolysaccharides. It is probable, however, that more than 80 per cent of the total body hexosamine is contained in plasma mucoproteins and tissue mucopolysaccharides.

Although many of these data are new, the methods used, unless otherwise specified, have been previously reported.[8-13]

Plasma Hexosamine and Stress

Clinically, the concentration of hexosamine in plasma is increased in many chronic diseases. It may also increase transiently following an acute clinical stress, as in a fracture, coronary thrombosis, attack of gouty arthritis,[11] or pneumonia.[14] Increased levels have been observed following experimental fractures or turpentine abscesses in the rat[15] and following blood-letting in the rabbit.[16] The response of plasma hexosamine levels to stress would therefore be similar in many ways to that of the sedimentation rate or C-reactive protein.

Distribution of Hexosamine in Plasma

The hexosamine (mostly glucosamine) concentration in plasma is normally about 80 mg. per cent.[11] Of this, only about 200 μg. per cent is in the form of low-molecular-weight dialyzable hexosamine compounds.[17] More than 99 per cent of the total hexosamine in plasma is incorporated in proteins.

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TABLE 1

PER CENT CONCENTRATION OF HEXOSAMINE IN NORMAL PLASMA GLOBULINS*

Source	α-1	α-2	β	γ	Total protein
Rat	4.42	2.55	1.87	0.87	1.73
Human	6.03	2.90	2.54	1.27	1.33

* Based on analyses of proteins separated electrophoretically on paper.

TABLE 2

DISTRIBUTION OF HEXOSAMINE IN PLASMA PROTEINS, EXPRESSED AS MILLIGRAMS PER CENT OF PLASMA*

Condition	Albumin† + α-1	α-2	β	γ	Total
Normal	22	18	16	22	78
Normal	23	20	9	17	71
Acute sepsis	47	46	20	25	138
Rheumatoid arthritis	48	45	16	32	141
Gouty arthritis:					
Before attack	31	27	20	28	106
During attack	39	46	21	28	134

* Based on analyses of proteins separated electrophoretically on paper.
† Purified albumin is essentially free of hexosamine.

Highly purified albumin is essentially free of hexosamine (less than 0.02 per cent).[17] Alpha-1 globulin or orosomucoid may contain as much as 12 per cent;[18] the remaining globulins contain less (TABLE 1). Following stress, the increase in plasma hexosamine levels may be related to changes in one or more of the globulins, but most generally the alpha-globulins (TABLE 2).

Endocrine Regulation of Plasma Hexosamine Levels

One of the earliest observed effects of hormones on blood hexosamine levels was reported in patients with acute disseminated lupus erythematosus who were treated with ACTH or cortisone.[19] Under the influence of either hormone, abnormally elevated serum hexosamine levels became lower; in this particular disease it was the gamma-globulin hexosamine that was primarily affected.[20]

In order to review systematically the role of the endocrine glands as mediators of the response of plasma hexosamine level to stress, rats were hypophysectomized, adrenalectomized, thyroidectomized, or gonadectomized, and their plasma hexosamine levels measured. Each operation, in itself, served as the stress agent. Intact sham-operated rats were used as controls. The increase in plasma hexosamine levels following adrenalectomy or gonadectomy was similar to that of the controls. Removal of the pituitary or thyroid glands, on the other hand, resulted in an augmented and sustained increase in plasma

hexosamine levels[12], greater than that seen in the sham-operated controls. In the thyroidectomized animals this was shown to be due primarily to an increase in gamma-globulin hexosamine.[10]

It appeared that in the rat, although the endocrines did not basically mediate the response of plasma hexosamine levels to stress, absence of the thyroid or pituitary glands profoundly influenced the degree of this response.

Local Connective Tissue Stress

Stress or trauma to connective tissue almost always results in local edema with an increase in the concentration of hexosamine at the injured site. This has been shown following injections of formalin, ammonia, alcohol, turpentine, tannic acid, and following experimental incisions or pinching of the skin.[21]

In order to study the effect of the endocrines on this response, 80- to 100-gm. male Sprague-Dawley rats were thyroidectomized, hypophysectomized, and adrenalectomized. Eight days postoperatively an experimental incision of 2 to 3 cm. was made on the right side of the dorsum of each rat and then sutured. The animals were sacrificed 2 days after the incision was made. Subcutaneous tissue was obtained from each animal from both the incised area and also from the unincised control area on the left side of the dorsum. After the water content of the tissues had been determined, the rats were defatted and hexosamine determinations were carried out according to methods described previously.[21] Of 12 thyroidectomized rats, only 6 were judged to be complete, as determined by I^{131} radioautographs;[22] the data are shown in TABLE 3. In each case the trauma of incision caused a local edema with an increase in water content. The greatest increase in hexosamine concentration was seen following thyroidectomy and the least following adrenalectomy. Having observed a similar augmented increase in the plasma hexosamine levels of thyroidectomized rats, it seemed important to investigate the normal distribution of hexosamine in connective tissue compounds.

TABLE 3

EFFECT OF EXPERIMENTAL INCISIONS ON THE LOCAL ACCUMULATION OF WATER AND HEXOSAMINE IN SUBCUTANEOUS CONNECTIVE TISSUE

Group	Area sampled	No. rats	Tissue water (per cent)	Hexosamine	
				Mg./100 gm. dry wt.	Change
Intact	Control	5	79.3 ± 0.6*	522 ± 14	+19%
	Incised		87.3 ± 0.6	646 ± 11	
Adrenalectomized	Control	5	77.7 ± 0.7	610 ± 23	+2%
	Incised		82.8 ± 1.0	622 ± 20	
Thyroidectomized†	Control	6	80.0 ± 0.6	518 ± 4	+40%
	Incised		85.2 ± 0.4	724 ± 15	
Hypophysectomized	Control	4	80.2 ± 1.0	618 ± 10	+19%
	Incised		86.1 ± 0.6	735 ± 30	

* Standard error of the mean.
† Completely thyroidectomized as determined by I^{131} radioautographs.

Connective Tissue Hexosamine and Plasma Proteins

It has been generally recognized that the plasma protein pool of the body is greater than can be accounted for by blood alone. Numerous studies have indicated that at least one half of the total plasma protein of the body is extravascular.[23] This has been demonstrated by the use of C^{14}-labeled proteins,[24-25] I^{131}-tagged albumin,[26] and protein-binding dyes such as Evans blue. One known extravascular depot of plasma proteins is the lymphatic fluid, in which concentrations vary from 2 to 4 per cent. Since the vascular and lymphatic systems are each closed systems, it is postulated that lymph plasma proteins arise as a result of their free flow through extracellular fluid from blood capillaries.[27]

Despite all these indirect observations and deductions, very little direct quantitative or qualitative work has been done on the noncollagenous soluble proteins of connective tissue ground substance. Gitlin *et al.* were able to show by direct analysis that myoalbumin was, in fact, interstitial fluid albumin, equal in amount to the total circulating albumin.[28] The localization of human plasma proteins in connective tissue and blood vessel walls has also been demonstrated histologically, using fluorescent human plasma protein antibodies in humans.[29] The existence of plasma proteins in ground substance of the aorta is suggested by the localization of Evans blue in it after intravenous injection in the dog.[30]

Only recently have more precise methods been applied to this problem. Harkness *et al.*, in their studies on collagen fractions of rabbit skin, separated a soluble noncollagenous protein that on electrophoresis was similar to those of rabbit plasma.[31] By calculation, these proteins represented 0.8 per cent of the wet cell-free weight of skin.

It has long been recognized that hyaluronic acid complexes with connective tissue proteins.[32] Although these proteins have not been clearly identified, many workers have referred to them as connective tissue mucoproteins. Consden[33] identified a mucoprotein fraction of connective tissue that contained hexosamine but no uronic acid. During the course of studies on connective tissue hexosamines we similarly observed a high hexosamine-uronic acid ratio, and concluded that there must be hexosamine-containing compounds other than mucopolysaccharides. Accordingly, fractionation procedures were undertaken further to characterize these hexosamine-containing mucoproteins.[9]

Fresh subcutaneous connective tissue was obtained from rats and pooled for analysis. In the first experiment, designed to isolate hyaluronic acid, the tissue was denatured in acetone and the mucopolysaccharides extracted with 5 per cent sodium acetate. The insoluble residue had a high hexosamine-uronic acid molar ratio (4.6:1), whereas the mucopolysaccharide extract had a molar ratio of about 1:1. Approximately 42 per cent of the hexosamine was in the mucopolysaccharide fraction, whereas 50 per cent remained with the denatured protein fraction. In the second experiment fresh connective tissue was extracted exhaustively with *p*H 8.6 Veronal buffer. This fraction contained hexosamine and was free of detectable uronic acid. Electrophoresis of this extract at *p*H 8.6 demonstrated protein peaks with mobilities similar

TABLE 4

ANALYSIS OF EXTRACTS OF UMBILICAL CORD AND COCK'S COMB, EXPRESSED AS CONCENTRATION (GRAMS) IN TOTAL TISSUE WATER

Saline extract	Cord	Comb
Hexoses	0.082	0.071
Hexosamines	0.106	0.136
Nitrogen	0.128	0.079
Hemoglobin	0.0005	0.0015
Protein (N × 6.25)	0.800	0.493

to those of normal rat serum albumin and globulins. By calculation, it could be shown that 50 per cent of the total connective tissue hexosamine was present in ground substance proteins having the same mobilities as plasma proteins.

Following the demonstration of plasma proteins in rat subcutaneous connective tissue it seemed worthwhile to examine tissues known to be rich in ground substance. To this end, fresh cock's combs and umbilical cords were used. The umbilical cords (13.7 gm.) were cut into short sections; the intermediate layers[1] of the combs (8.7 gm.) were carefully dissected. Each tissue was rinsed under tap water, ground in a Waring Blendor with 0.9 per cent saline, and permitted to stand for 24 hours at 4° C. The tissues were then incubated at 37° C. for 20 min. with 1000 viscosity-reducing units (V.R.U.) of hyaluronidase, and filtered with Celite filter aid. The residues were re-extracted with saline, and all the filtrates were pooled, dialyzed, and lyophilized to dryness. The cord extract weighed 326 mg., the comb extract 117 mg.

The fresh cords contained 92.2 per cent water, the combs 90.0 per cent. All data are expressed relative to total tissue water. Hexosamine, hexose, nitrogen, and hemoglobin analyses are shown in TABLE 4. The cord and comb data were generally similar. Concentrations of cord and comb proteins, as represented by these extracts, were 0.80 per cent and 0.49 per cent, respectively. The concentration of hexosamine was 10 to 15 times higher than would be expected if contributed only by plasma proteins. It is likely that depolymerized but nondialyzable hyaluronic acid was present in both extracts, since these tissues are rich in this mucopolysaccharide.[34, 35] Total reducing values were only slightly higher than would be expected from the amount of proteins present. Hemoglobin values were very low, indicating negligible contamination by intravascular blood.

FIGURE 1 demonstrates typical patterns obtained by zone electrophoresis (*p*H 8.6 Veronal buffer) using these cord and comb extracts. In both instances a large peak with a mobility similar to that of normal serum albumin was present.* In addition, the cord extract demonstrated peaks coincident in mobility with those of normal beta- and gamma-globulins. The globulin fractions were less clearly defined in the comb extract.

* The mobility of the albumin in the extracts was slightly less than that of the serum albumin. This was probably due to an artifact, since mixtures of extracts and serum had a single albumin peak (see FIGURE 2).

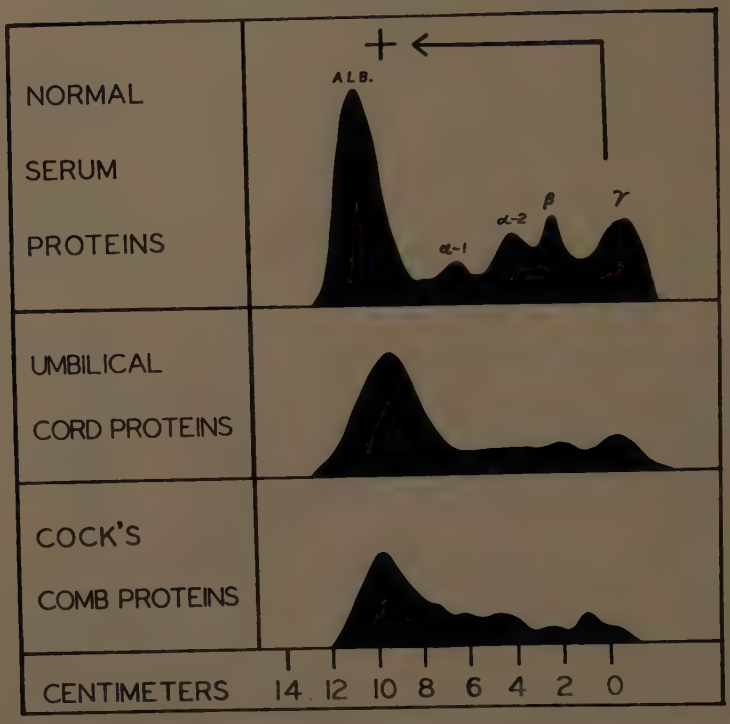

FIGURE 1. Electrophoretic distribution of proteins in saline extracts of umbilical cord and cock's comb.

In another experiment fresh umbilical cords were extracted with saline (0.9 per cent). When the extract was dialyzed, a precipitate formed. The water-soluble supernatant was subsequently made to 10 per cent and 18 per cent with sodium sulfate, and no further precipitation occurred. The 18 per cent sodium sulfate soluble fraction and water-insoluble fraction were separated electrophoretically on paper in *p*H 8.6 Veronal buffer (FIGURE 2). The water-insoluble fraction showed a mobility similar to that of gamma-globulin, and the 18 per cent sodium sulfate-soluble fraction was similar to albumin. Using the same technique, we have similarly demonstrated proteins in perirenal fat of normal and thyroidectomized guinea pigs that were electrophoretically similar to plasma proteins.[17]

Considerable evidence is now accumulating indicating the presence of plasma proteins in many connective tissue sites.

Of great interest is a case of clinical myxedema in which Bauman *et al.*, using an I^{131} technique, demonstrated an increased extravascular pool of plasma proteins.[36] By using I^{131}-labeled plasma proteins, as well as by direct analysis, Cohen has shown that the sex skin of the baboon is rich in plasma proteins.[3] During the follicular phase of the menstrual cycle the perineum swells, the protein content increases, the plasma protein pool is increased, and plasma

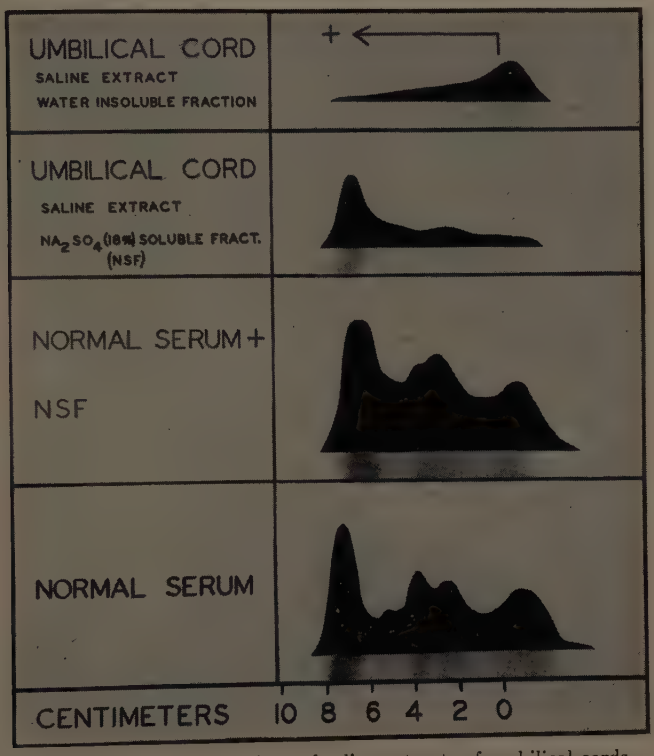

FIGURE 2. Electrophoresis of fractions of saline extracts of umbilical cords.

proteins are synthesized more rapidly. Plasma proteins in perineal fluid were identified by ultracentrifugation and electrophoresis. Cooper and Johnson have more recently demonstrated proteins similar to plasma proteins in saline extracts of bovine hide, using electrophoresis and sedimentation studies.[37] Utilizing immunological methods and electrophoresis, plasma proteins have also been demonstrated in rabbit and rat skin, and tendon of rat, rabbit, and ox.[25]

Summary

Hexosamines are widely distributed in the animal body, existing predominantly as components of plasma proteins and tissue mucopolysaccharides.

Blood plasma hexosamine levels are increased with acute or chronic stress. This increase may be profoundly modified by hormones of the thyroid and adrenal glands, or by the absence of the thyroid or pituitary glands.

The tissue concentration of hexosamine is also increased with local stress (injury) and may also be modified by the absence of either the adrenal or thyroid gland.

Studies of the distribution of hexosamine in connective tissue revealed an abundance of plasma proteins in connective tissue ground substance.

Plasma proteins have now been demonstrated by direct methods in the connective tissue ground substance of rat subcutaneous tissue, umbilical cord, cock's comb, perirenal fat of the guinea pig, sex skin of the baboon, skin of the rabbit, rat, and cow, tendon of rabbit, rat, and ox, and rabbit muscle.

It is most probable that plasma proteins are normal components of all loose connective tissue, including the walls of blood vessels. Inasmuch as plasma protein hexosamine levels may be profoundly influenced by stress and the endocrine system, it may be presumed that similar influences prevail in regulating plasma proteins of connective tissue. It is probable that the plasma proteins in blood vessel walls, as regulated by hormones and stress, play a vital role in the genesis of arteriosclerosis.

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